

AP-1 PROTEINS REGULATE AUTOPHAGY IN SKELETAL MUSCLE
PROGENITOR CELLS

TRACEY EDWARDS

A THESIS SUBMITTED TO
THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

GRADUATE PROGRAM IN BIOLOGY
YORK UNIVERSITY
TORONTO, ONTARIO

April 2019

© Tracey Edwards, 2019

Abstract

Autophagy has been observed to be a key regulator in myogenesis, maintenance of quiescence in satellite cells (SC) and regenerative capacity of skeletal muscle. Activator protein-1 (AP-1) transcription factors have been proteins of interest in autophagy regulation in various cancers due to the established roles of AP-1 in cellular proliferation, death and survival. However, AP-1 involvement in the regulation autophagy in skeletal muscle progenitor cells has not been extensively assessed. AP-1 protein c-Jun is expressed in paired box gene 7 (Pax7) positive satellite cells. AP-1 proteins c-Jun and JunB regulate autophagy. Changes to c-Jun expression lead to changed autophagosome content in C2C12 cells in both growth conditions and induced autophagy conditions. Additionally, c-Jun knockdown altered autophagosome content in cultures containing Pax7⁺ and Pax7⁻ cells. AP-1 protein c-Jun appeared to be regulated by mTor. AP-1 proteins act as autophagy regulators dependent on cell type and autophagy induction method.

Acknowledgements

First and foremost, I would like to thank Dr. McDermott for taking me on as a graduate student and allowing me this opportunity to conduct research. I have learned so much during this short time, and this experience has truly nurtured my curiosity.

Secondly, I would like to thank all the members of our lab group for their encouragement and support. Each person in the lab has helped me throughout these years here and have created a friendly and encouraging research environment. I would especially like to thank Dan Dan for spending countless hours teaching me methods essential to completing my experiments. I would also like to thank Michael, my fellow Master's candidate, for always being a person to bounce ideas off of and to help troubleshoot problems as they arose.

Lastly, I would like to thank my family and friends for supporting me throughout this entire degree.

Table of Contents

ABSTRACT	II
ACKNOWLEDGEMENTS.....	III
TABLE OF CONTENTS	IV
LIST OF ABBREVIATION	VII
LIST OF FIGURES	X
LITERATURE REVIEW	1
ORIGIN OF MUSCLE: FROM EMBRYOGENESIS TO POST-NATAL DEVELOPMENT	1
Figure 1: Schematic of skeletal muscle formation and differentiation.	3
THE ROLE OF PAX3 AND PAX7 IN EMBRYONIC MYOGENESIS	4
PAX7 POSITIVE CELLS ARE CRITICAL FOR POST-NATAL INJURY RESPONSE	5
AUTOPHAGY MAINTAINS HOMEOSTASIS	7
Figure 2: Schematic representation of autophagy.....	8
INITIATION OF AUTOPHAGY	9
PHAGOPHORE FORMATION AND ELONGATION RESULTING IN AUTOPHAGOSOME.....	9
REGULATION OF AUTOPHAGY	11
AUTOPHAGY REGULATES MYOGENESIS	13
ACTIVATING PROTEIN-1	14
ACTIVITY OF AP-1 PROTEINS IS HIGHLY DIVERSE.....	16
AP-1 IN SKELETAL MUSCLE DIFFERENTIATION	17
RELATIONSHIP BETWEEN AP-1 AND AUTOPHAGY	18

STATEMENT OF PURPOSE.....	20
Figure 3: Proposed relation of AP-1 proteins to autophagy.....	21
INTRODUCTION.....	22
MATERIAL AND METHODS.....	24
RESULTS	30
C-JUN IS EXPRESSED IN SATELLITE CELLS.....	30
Figure 4: c-Jun localization in satellite cells on myofiber.....	31
ENDOGENOUS C-JUN REDUCES LC3II AUTOPHAGOSOME CONTENT IN PROLIFERATIVE CONDITIONS IN C2C12 MYOBLASTS	32
Figure 5: Reduction of endogenous c-Jun leads to an increase in LC3II in C2C12 myoblasts.	33
REDUCTION OF ENDOGENOUS C-JUN IN PRIMARY SKELETAL MUSCLE CULTURE REDUCES LC3II AUTOPHAGOSOME CONTENT.....	34
Figure 6: Reduction of c-Jun in primary skeletal muscle culture decreases LC3II autophagosome content.	35
REDUCTION OF ENDOGENOUS C-JUN IN C2C12 MYOBLAST HAS NO EFFECT ON RAPAMYCIN INDUCED AUTOPHAGY	36
Figure 7: Reduction of c-Jun has no effect on Rapamycin induced autophagy autophagosome content.	
ECTOPIC EXPRESSION OF C-JUN HAS UNIQUE EFFECTS ON AUTOPHAGOSOME CONTENT DEPENDENT ON MODE OF AUTOPHAGY INDUCTION	37

Figure 8: Ectopic expression of c-Jun regulates autophagosome content dependent on autophagy induction method in C2C12 myoblasts.	39
AP-1 PROTEINS HAVE UNIQUE EFFECTS ON INDUCED AUTOPHAGY IN C2C12 MYOBLASTS.....	40
Figure 9: Jun proteins reduce induced autophagy.	41
DISCUSSION	42
Figure 10: AP-1 protein c-Jun in relation to autophagy induction mechanisms.	48
CONCLUSIONS AND FUTURE WORK.....	49
REFERENCES.....	52
APPENDIX: EXTENDED MATERIALS AND METHODS	62
PRIMARY SKELETAL MUSCLE ISOLATION AND PRE-PLATING	62
POLYETHYLENEIMINE TRANSFECTION IN C2C12 CELLS	65
CALCIUM PHOSPHATE (CaPO ₄) siRNA TRANSFECTION IN C2C12 CELLS.....	65
LIPOFECTAMINE 2000 TRANSFECTION PROTOCOL OF PRIMARY SKELETAL MUSCLE	67
WESTERN BLOT ANALYSIS PROTOCOL.....	68
SKELETAL MUSCLE FIBER ISOLATION	72

List of Abbreviations

AP-1 – Activating protein-1

AKT – Protein kinase B

ATG – Autophagy-related genes

ATP – Adenosine triphosphate

BAG3 – Bcl-2 associated athanogene 3

Bcl-2 – B-cell lymphoma 2

Bim – Bcl-2-like protein 11

BMP – Bone Morphogenetic Protein

Bnip3 – Bcl-2 interacting protein 3

b-ZIP – Basic region-leucine zipper

c-Met – Tyrosine-protein kinase Met also known as hepatocyte growth factor receptor (HGFR)

DMD – Duchenne Muscular Dystrophy

ERK 1/2 – Extracellular signal-regulated kinases 1/2

FGF – Fibroblast growth factor

FIP200 – FAK family- interacting protein of 200kD

FOXO3 – Forkhead box O3

HGF – Hepatocyte growth factor

JNK – c-Jun N-terminal kinase

LAMPs – Lysosomal- associated membrane proteins

LC3 -- Microtubule-associated protein 1A/1B light chain 3B protein

IRE1 – Inositol-requiring enzyme 1

MAPKs – Mitogen-activated protein kinases

MCK – Muscle Creatine Kinase

MEF2 – Myocyte Enhancer Factor-2 proteins

MEFs – Mouse embryonic fibroblasts

MRF – Myogenic Regulatory Factors

Mrf4 – Myogenic Regulatory Factor 6, also known as MYF6 and Herculin

Myf5 – Myogenic Factor 5

MyHC – Myosin Heavy Chain

MyoD – Myogenic Differentiation 1

MyoG – Myogenin

mTORC—Mammalian Target of Rapamycin Complex

mTOR—Mammalian Target of Rapamycin

Pax3 – Paired box gene 3

Pax7 – Paired box gene 7

PE -- phosphatidylethanolamine

PI3K or PtdIns3K -- Class III phosphatidylinositol 3-kinase also known as hVps34

SC – Satellite cells

SF – Scatter Factor

Shh – Sonic Hedgehog

siRNA – Silent interfering RNA

SV40 – Simian Vacuolating Virus 40

TA – Tibialis Anterior

tLC3 – Tandem tagged LC3

TPA cis element – 12-O-tetradecanoylphorbol-13-acetate response element

TorC1 – Target of Rapamycin complex 1

ULK 1/2 – Unc-51 like kinase-1/2

UVRAG – UV irradiation resistance- associated gene

Wnt – Wingless/Integrated

List of Figures

Figure 1: Schematic of skeletal muscle formation and differentiation

Figure 2: Schematic representation of autophagy

Figure 3: Proposed relation of AP-1 proteins to autophagy

Figure 4: c-Jun localization in satellite cell on myofibers

Figure 5: Reduction of endogenous c-Jun leads to an increase in autophagy in C2C12 myoblasts

Figure 6: Reduction of c-Jun in primary skeletal muscle culture decreases autophagy

Figure 7: Reduction of c-Jun has no effect on Rapamycin induced autophagy

Figure 8: Ectopic expression of c-Jun regulates autophagy dependent on autophagy induction method in C2C12 myoblasts

Figure 9: Jun proteins reduce induced autophagy

Figure 10: AP-1 protein c-Jun in relation to autophagy induction mechanisms

Literature Review

Origin of muscle: from embryogenesis to post-natal development

Each day the majority of our functional movements such as grasping, locomotion and even diaphragmatic breathing require the function of skeletal muscles. Skeletal muscle is formed during myogenesis and is derived from somites (Buckingham et al. 2003). During embryogenic myogenesis, the hypaxial dermomyotome gives rise to the muscles of the body and limbs, while the paraxial mesoderm and the prechordal mesoderm give rise to the head muscles (Buckingham et al. 2003). The paraxial mesoderm itself arises after gastrulation occurs in the blastopore (Chal and Pourquié 2017). Somites are formed from the paraxial mesoderm which segments to form the somites during somitogenesis (Buckingham et al. 2003; Chal and Pourquié 2017). Segmentation of the somites is highly dependent on the morphogen gradients of Wingless/Integrated (Wnt), fibroblast growth factor (FGF) and retinoic acid (Bentzinger, Wang, and Rudnicki 2012). The somites become compartmentalized to form both a dorsal epithelial dermomyotome and a ventral mesenchymal sclerotome (Chal and Pourquié 2017). The ventral sclerotome gives rise to skeletal structures while the dermomyotome produces skeletal muscle (Chal and Pourquié 2017). Somites then further divide into both lateral and dorsal compartments. Hypaxial muscles will be formed from cells located in lateral somite while medial cells form the paraxial muscle (Olivera-Martinez et al. 2000; Ordahl and Le Douarin 1992).

The inhibition of Bone Morphogenetic Protein (BMP) along with the Wnt signaling is essential to both dermomyotome specification and maintenance (Hirsinger et al. 1997).

The cells of the dermomyotome express Paired box gene 3 (Pax3) Pax3 along with Paired box gene 7 (Pax7). Both the hypaxial ventrally located lips and the dorsally located epaxial lips of the dermomyotome further mature into the myotome which is the primitive muscle structure containing cells which express Myogenic differentiation 1 (MyoD) and Myogenic factor 5 (Myf5), both which are Myogenic Regulatory Factors (MRFs) (Figure 1a) (Bentzinger, Wang, and Rudnicki 2012). With the expression of MyoD and Myf5 initiated in the cells, cells are now committed to the myogenic lineage and are considered myoblasts. The maturation of the dermomyotome into the myotome is highly dependent on Sonic Hedgehog (Shh) signaling from the notochord and the floor plate of the neural tube which causes the upregulation of MRFs, MyoD and Myf5 along with the downregulation of Pax3 (Bentzinger, Wang, and Rudnicki 2012). Cells originating in the dermomyotome expressing Pax3 will also migrate to limb sites and further differentiate to form myofibers with an upregulation in expression of both MyoG and MCK, marking late differentiation (Bentzinger, Wang, and Rudnicki 2012).

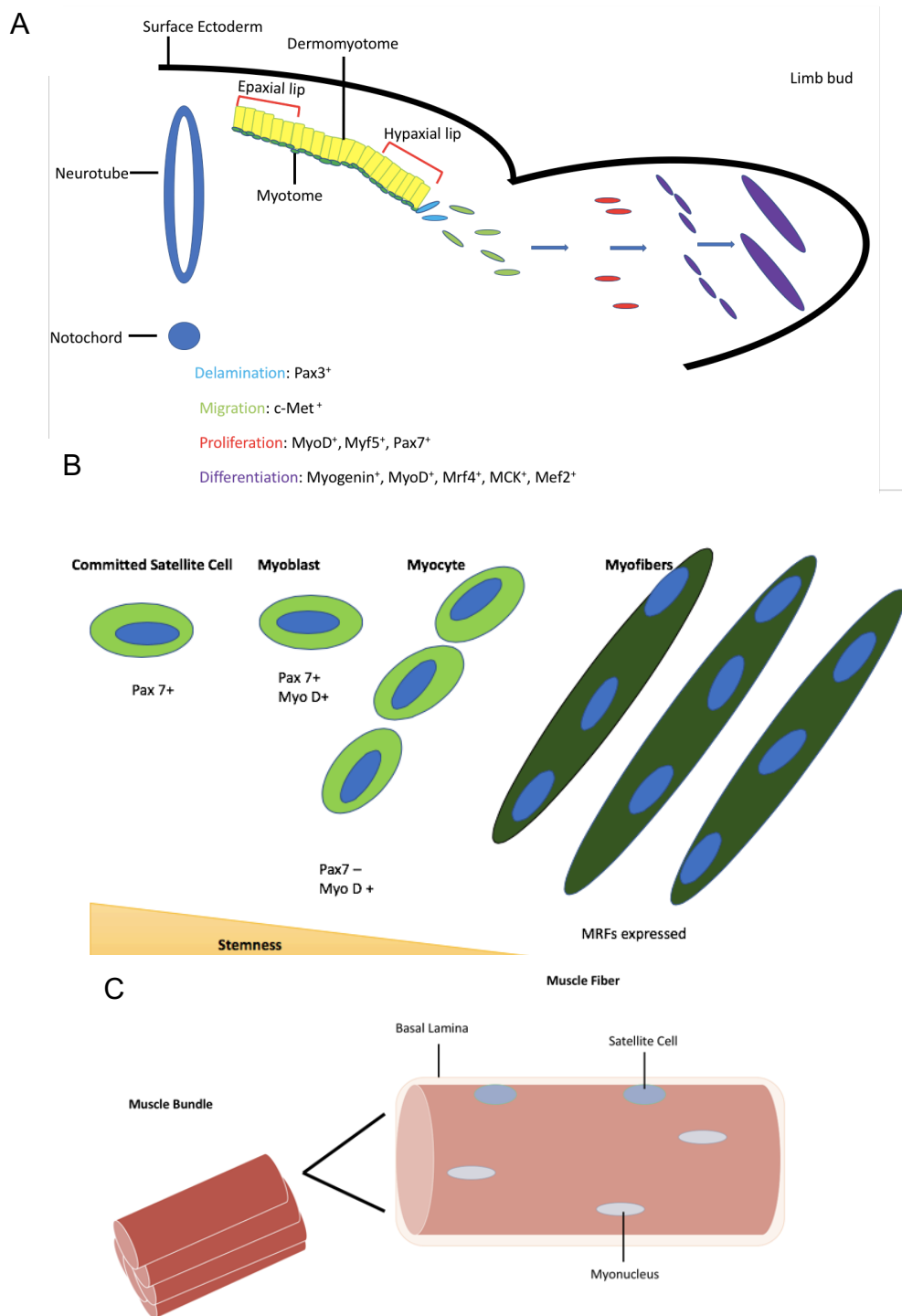


Figure 1: Schematic of skeletal muscle formation and differentiation.

(A) Following somitogenesis cells of the dermomyotome migrate and mature to form the myotome and eventually muscle and eventually committed myogenic lineage cells. The myogenic cells progress to form myofibers. **(B)** The progression of embryonic progenitor cells to matured myotubes during muscle differentiation. As myogenic cells lose the expression of Pax7 they also lose their stemness and progress into the final differentiated state. **(C)** Schematic of mature muscle fiber with satellite cell. Images adapted from (Bentzinger et al. 2012; Buckingham et al. 2003).

The role of Pax3 and Pax7 in Embryonic Myogenesis

Cells migrate from the dermomyotome to form limb muscles due to Pax3 signaling (Bentzinger, Wang, and Rudnicki 2012). Pax3 is a paired-homeobox transcription factor (Bentzinger, Wang, and Rudnicki 2012). Expression of Pax 3 transcription factor induces the transcription of the Tyrosine- protein kinase Met (c-met). C-met interacts with scatter factor (SF) and hepatocyte growth factor (HGF), dictating the migratory route for the somite derived cells (Buckingham et al. 2003; Dietrich et al. 1999). Following migration, the cells migrating from the dermomyotome then express MyoD and Myf5 and proliferate in the limb area (Buckingham et al. 2003; Delfini et al. 2005). The expression of Myf5 correlates with the downregulation of Pax3 expression (Bober et al. 1994; Chal and Pourquie, 2017). With the expression of MyoD and Myf5 initiated in the cells, they are now committed to the myogenic lineage and are myoblasts. Proliferation of these cells in the forming limb has been attributed to signaling through fibroblast growth factor (FGF) receptors (Buckingham et al. 2003; Edom-Vovard, Bonnin, and Duprez 2001). These myoblasts further mature and differentiate into myofibers. Differentiation in the muscle progenitor cells is dependent on the expression of Myogenin (MyoG) and Myocyte enhancer factor-2 (Mef2) (Buckingham et al. 2003). During the perinatal period, primary and secondary muscle fibers form (Buckingham et al. 2003). During the primary phase, myofibers are derived from Pax3 positive cells (Horst et al. 2006; Hutcheson et al. 2009; Otto, Schmidt, and Patel 2006). These primary fibers are the supporting features for the limb muscles and myotomes which myofibers from the secondary phase will be formed upon (Murphy and Kardon 2011).

During the secondary phase of myogenesis, a cohort of the Pax3 positive cells begins to upregulate Pax7 expression (Chal and Pourquié 2017). The expression of Pax7 coincides with the downregulation of Pax3 in the myogenic progenitor cells (Chal and Pourquié 2017). This secondary phase results in the production of myoblast positive with Pax7 expression. Pax7 expression along with Myf5 decreases during early differentiation and expression ceases differentiation furthers to myocyte stage (Bentzinger, Wang, and Rudnicki 2012). Further differentiation of myocytes into myofibers is marked by the expression of MyoD, MyoG as well as Mrf4 (Figure 1b) (Bentzinger, Wang, and Rudnicki 2012). The primary phase as well results in the establishment of a pool of Pax7 positive progenitor cells (Chal and Pourquié 2017). This pool of Pax7 progenitor cells derived from the Pax3 positive population are known as satellite cells. The Pax7 expressing satellite cells migrate to lay on the newly established myofibers (Yin, Price, and Rudnicki 2013). These cells reside in a specific environment, laying on top of the sarcolemma within a continuous layer of basal lamina which surrounds the myofiber (Yin, Price, and Rudnicki 2013). These cells maintain a quiescent state and are critical for regeneration of muscle in post-natal development (Chal and Pourquié 2017; Seale et al. 2000).

Pax7 Positive Cells Are Critical for Post-Natal Injury Response

Following injury, myogenesis in adults is dependent on progenitor cells to produce new myofibers on existing muscle fibers (Bentzinger, Wang, and Rudnicki 2012). The production of the new progenitor cells is dependent on satellite cells. Pax7 positive satellite cells remain in a quiescent state and, upon injury undergo asymmetric

proliferation to form cells expressing both Pax7 and MyoD cells that will eventually differentiate into new myofibers, losing Pax 7 expression, as well as cells that maintain a stem cell pool which will continue to express Pax7 (Cheung and Rando 2013). Cells bound for differentiation enter a committed stage in which expression of both Pax7 and Myf5 are detected (Bentzinger, Wang, and Rudnicki 2012).

Following the proliferation of myoblast, Pax7 expression along with Myf5 decrease during early differentiation and expression of both Pax7 and Myf5 ceases as differentiation furthers to myocyte stage (Bentzinger, Wang, and Rudnicki 2012).

Further differentiation of myocytes into myofibers is marked by the expression of MyoD, MyoG as well as Mrf4 (Bentzinger, Wang, and Rudnicki 2012). Pax7 is a member of the homeodomain family transcription factors (Chi and Epstein 2002). The Pax7 gene contains three conserved domains: pair domain, octapeptide and a paired-type homeodomain, and is a member of the paired-homeobox gene family (Jostes et al., 1991). Pax7 affects postnatal growth through its regulation of myogenic satellite cell specification, modification and maintenance (Olguin et al. 2007; Relaix et al. 2006; Seale et al. 2000). In the absence of Pax7 expression, induced through Cre-lox induced knock out, hind limb muscles are unable to regenerate following injury (Lepper, Partridge, and Fan 2011). It has been observed that Pax7 induction is essential for myogenic satellite cell specification in mice (Seale et al. 2000). The Pax7 positive satellite cell population undergoes a significant depletion during post-natal development from approximately 30% of muscular cells to 2% of muscular nuclei after two months of development (Gibson and Schultz 1983). The decline in satellite cells has been linked to macroautophagy (García-Prat et al. 2016).

Autophagy maintains Homeostasis

Autophagy is a general term to describe the clearing of cytosolic contents from a cell (Klionsky 2007). The process itself is characterized by the initiation of a phagophore, the elongation of the phagophore to form the double membraned autophagosome and the fusion of the mature autophagosome with a lysosome to form the autolysosome which degrades its internal contents (Klionsky 2007). Three distinct forms of autophagy have been observed, 1) chaperone-mediated autophagy, 2) microautophagy and 3) macroautophagy. Macroautophagy (referred to as autophagy for remainder) has been observed to play a role in both differentiation of skeletal muscle and the maintenance of quiescence in satellite cells and has been studied in both tumor survival and neurodegeneration (Fiacco et al. 2016; García-Prat et al. 2016; Rubinsztein 2006; Rubinsztein et al. 2007). Autophagy involves the degradation of cytoplasmic components including organelles and proteins via delivery of a specialized vacuole to lysosomal digestion (Mehrpour et al. 2010). Autophagy is a conserved process in eukaryotic cells occurring both in normal conditions and during periods of cellular stress (Mehrpour et al. 2010). Autophagy acts in normal conditions to prevent cytotoxicity by removing damaged organelles as well as proteins which may aggregate within the cell as seen in neurodegeneration (Rubinsztein 2006). However, during stress inducing conditions such as nutrient starvation, autophagy plays a role in the recycling of amino and fatty acids in order to preserve both adenosine triphosphate (ATP) levels and help to restore metabolism, allowing cell survival (Kuma et al. 2004; Mehrpour et al. 2010).

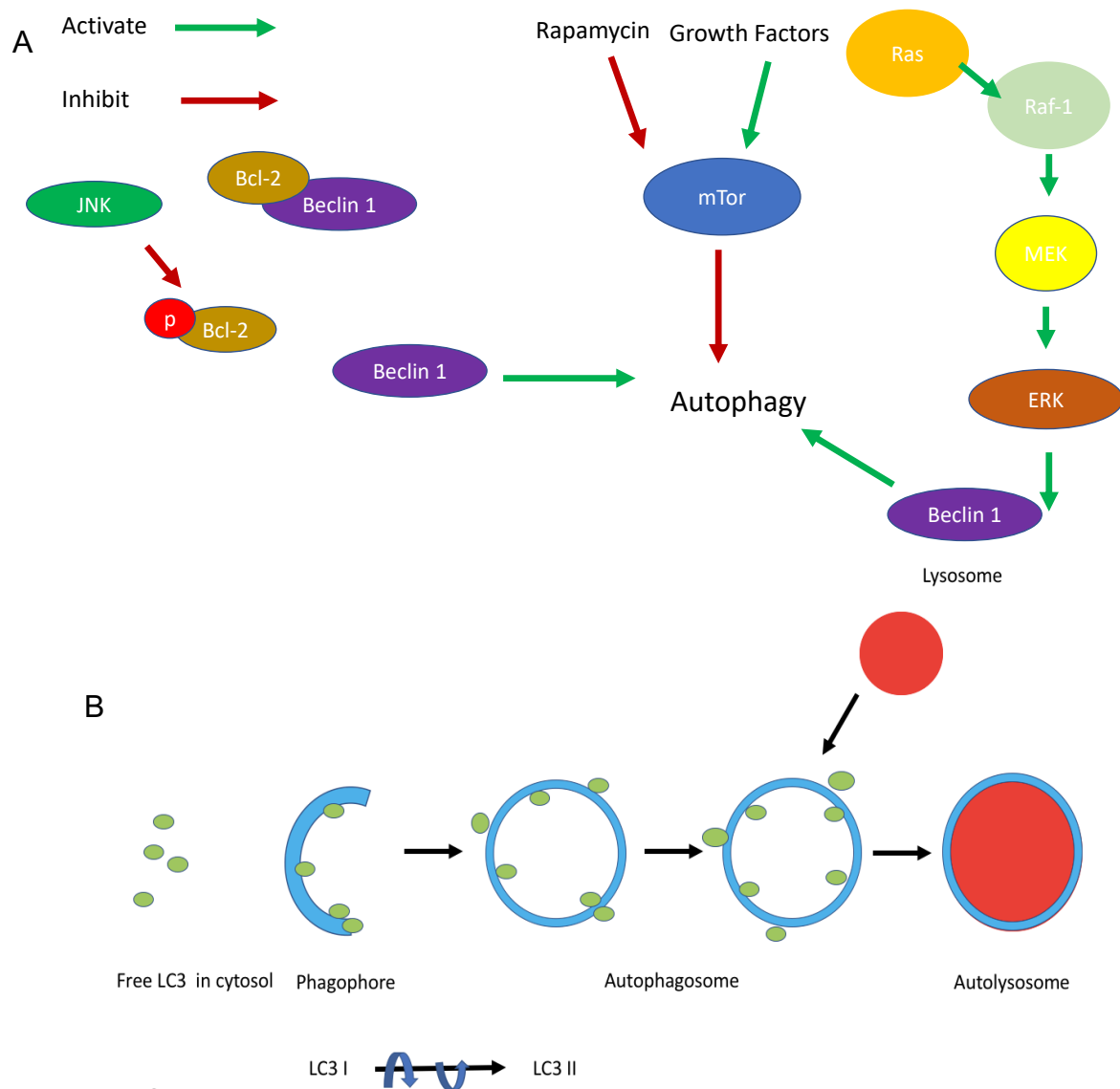


Figure 2: Schematic representation of autophagy.

(A) A schematic of autophagy induction pathways. Autophagy can be inhibited by mTOR in its activated state. Rapamycin inhibits mTOR leading to the induction of autophagy. Autophagy can also be induced through Beclin-1- a key component in the autophagy induction complex. Beclin-1 is inhibited by Bcl-2 and can be indirectly activated by JNK through the phosphorylation of Bcl-2 leading to its dissociation from Beclin-1. Ras also acts through its downstream target ERK to regulate Beclin-1. **(B)** A representation of autophagy structures. An initial phagophore elongates with the incorporation of LC3 in the cytosol leading to the formation of an autophagosome. The autophagosome and its contents are degraded upon fusion with a lysosome and formation of an autolysosome. Adapted from Mehrpour et al. 2010.

Initiation of Autophagy

Autophagy related genes (Atg) have been characterized in yeast, and many have mammalian homologs. The initiation of autophagy begins with the autophagosome formation (Mehrpour et al. 2010). In yeast, the formation of the autophagosome is controlled by an Atg 1: Atg 13: Atg 17 complex (Mehrpour et al. 2010). In mammals the initiation complex is composed of the Atg 1 homologs unc-51 like kinase-1 (ULK1) and unc-51 like kinase-2 (ULK2), and Atg17 homolog FIP200 which interact with the mammalian form of Atg forming the stable ULK:ATG13:FIP200 complex (Hosokawa et al. 2009; Mehrpour et al. 2010). The ULK:ATG13:FIP200 complex is stable in basal conditions (Mehrpour et al. 2010). The ULK:ATG13:FIP200 complex then interacts with Atg 10, which localizes the complex to the phagophore and aids the in stabilization of Atg13 expression (Mehrpour et al. 2010). This interaction between the mammalian complex and Atg 10 occurs in all conditions – basal and stressed conditions (Mehrpour et al. 2010).

Phagophore Formation and Elongation Resulting in Autophagosome

Class III phosphatidylinositol 3-kinase (PI3K), also known as hVps34 complex is critical for the assembly of the phagophore. The PI3K complex is composed of III PI3K along with p150, Beclin-1 (Itakura et al. 2008). The PI3K phagophore critical complex interacts with previously described ULK:ATG13:FIP200 to regulate the beginning of autophagy. Following the initiation of the phagophore, the Beclin-1:PI3K:Atg14L complex and other Atg proteins recruit a complex composed of Atg proteins Atg12-Atg5:Atg16L as well as

microtubule-associated protein 1A/1B light chain 3B protein (LC3) (Mehrpour et al. 2010). Atg 16L and LC3 have both been shown to be critical for the elongation of the phagophore (Lee and Lee 2016; Nakatogawa et al. 2009). During this process, Atg 12 is conjugated to Atg5 by Atg10. Along with the conjugation of Atg 12, LC3 undergoes transformation through cleavage by Atg4 (Mehrpour et al. 2010). LC3 is cleaved at the C terminus resulting in a glycine residue at the start of autophagy (Mehrpour et al. 2010). The conjugated LC3, known as LC3 II localizes on both the inner and outer membranes of the newly formed autophagosome formed at the end of elongation; unconjugated LC3 localizes in the cytoplasm (Mehrpour et al. 2010).

Although non-canonical pathways to autophagosome formation have been discovered, they have not been extensively investigated (Mehrpour et al. 2010). These pathways occur only with some of the previously described Atg proteins.

Upon the formation of the autophagosome, the autophagosome can fuse with an endosome forming an amphisome, prior to fusion with a lysosome (Seglen et al. 1990; Mehrpour et al. 2010). Upon the autophagosome fusion with a lysosome it reaches its final stage of maturation forming the autolysosome. The regulation of these fusion steps is dependent on various proteins which can either upregulate or downregulate the transport of autophagosomes to endosomes and lysosomes (Mehrpour et al. 2010).

The manipulation of autophagosome trafficking and fusion to a lysosome effects the “autophagic flux” of the cell. The Beclin-1 binding protein Rubicon has been observed to downregulate the trafficking of the autophagosomes when complexed with Beclin-1, PI3K, and UV irradiation resistance-associated gene protein (UVRAG) (Matsunaga et

al. 2009). UVRAG has been observed to upregulate the trafficking events of autophagosomes (Matsunaga et al. 2009).

Lysosomal-associated membrane proteins (LAMPs) also play a role in the final stages of autophagy. LAMPs are endosome/lysosome transmembrane proteins (Eskelinen, Tanaka, and Saftig 2003). In mouse origin fibroblasts, only fibroblasts which are deficient for both LAMP-1 and LAMP-2 were observed to have blocked late stage autophagy (Tanaka et al. 2000).

The actual movement of autophagosomes in later stages of autophagy during basal conditions is also dependent on the role of microtubules. Through the use of nocodazole in mouse hepatocytes, a microtubule destabilizer, a decrease in fusion events between autophagosomes and lysosomes has been observed (Bechet et al. 2005). In instances of starvation, the end stage fusion events required for the formation of the autolysosome can be microtubule independent (Fass et al. 2006). In instances of starvation, lysosomes can fuse with autophagosomes that are in close proximity (Fass et al. 2006).

Regulation of Autophagy

The process of autophagy is highly conserved; however, the induction of this process can occur through many different pathways. The TOR pathway has been extensively studied in both yeast and mammalian models.

TOR complex 1 (TORC1), in mammals known as mammalian TOR complex1 (mTORC) acts to suppress autophagy in non-starvation states (Stokoe et al. 1997). The mTORC1, referred to as mTOR for the remainder, is a nutrient signaling pathway in the

regulation of autophagy. Rapamycin inhibits mTOR1, leading to the induction of autophagy in non-starvation conditions (Stokoe et al. 1997). It has also been observed that mTOR activation can occur through the stimulation of PI3K (He and Klionsky 2009). Upon the detection of amino acids, PI3K is stimulated and activates mTOR leading to autophagy inhibition. mTOR primarily acts as an autophagy inhibitor, only through its suppression is mTOR mediated autophagy able to be initiated.

Growth factors can also inhibit or induce autophagy through the Ras signaling pathway (He and Klionsky 2009). Through Ras, growth factor signals are conveyed to class I PI3K which inhibits autophagy in mouse embryonic fibroblasts (MEFs) (Furuta et al. 2004). Growth factors act as ligands for tyrosine kinase receptors and the signal is transduced by Ras to class I PI3K (Furuta et al. 2004). However, Ras can also convey the growth factor signal from the tyrosine kinase receptor to Raf-1/ Mitogen-activated protein kinases (MAPKs) in human colon cancer cells. Raf-1 is a sensor for amino acids and leads to the inhibition of autophagy through the downregulation of the downstream targets MAPK and extracellular signal-regulated kinases 1/2 (ERK 1/2) (Pattingre, Bauvy, and Codogno 2003). This allows for the preservation of proteins in instances of amino acid deprivation. ERK promotes autophagy through the regulation of Beclin-1 protein in cells exposed to X-ray radiation and nutrient starved cells (Kochetkova et al. 2017; Wang et al. 2009).

c-Jun N-terminal kinase (JNK) has been observed to regulate autophagy in multiple contexts. For example, JNK has been observed as a factor in the regulation of LC3 lipidation (Ogata et al. 2006). Instances of increases in misfolded proteins causes ER stress signals to activate inositol-requiring enzyme 1 (IRE-1), leading to the activation of

its downstream target JNK (Ogata et al. 2006). Additionally, JNK has been observed to be a post-translational regulator of autophagy. JNK phosphorylates B-cell lymphoma 2 (Bcl-2), an inhibitor of autophagy in both instances of nutrient starvation and ceramide treatment in what cell types (Pattingre et al. 2009). The phosphorylation of Bcl-2 occur at Thr69, Ser70 and Ser87 leading to a hyperphosphorylation of the inhibitor which can no longer bind to autophagy initiator Beclin-1, allowing Beclin-1 to induce autophagy (Pattingre et al. 2009).

The pathway which induces the initiation of autophagy is highly dependent on the stressor and the tissue type. Nutrient signaling, insulin, growth factors, endoplasmic reticulum stress, oxidative stress and pathogen infection all can stimulate the induction of autophagy through many different mechanisms (He and Klionsky 2009).

Autophagy Regulates Myogenesis

Along with autophagy's role in disease and maintaining homeostasis, autophagy has also been shown be a factor in both post-natal myogenesis and regeneration.

Autophagy maintains the regenerative capacity of satellite cells (García-Prat et al. 2016). In mouse derived Pax7 positive satellite cells a higher amount of autophagy was observed, as well during transplantation experiments, the induction of autophagy in geriatric derived satellite cells with lower levels of autophagy helped restore quiescence post-transplantation (García-Prat et al. 2016). Reduced autophagic flux in humans is also associated with aged patients (García-Prat et al. 2016). The age-related reduction in autophagic flux correlates with a regenerative impairment typically seen in later stages of adult life. Autophagy as a regulator of satellite cell maintenance of

regenerative capacity has also been observed in disease models of Duchenne muscular dystrophy (DMD) (Fiacco et al. 2016). In the early stages of DMD, patients are able to counter the degenerative effects due to satellite cell mediated regeneration (Collins et al. 2005). This enhanced regenerative capacity in DMD has been associated with an increase in autophagic flux in DMD models tibialis anterior (TA) muscles compared to wild type in the early stages of the disease (Fiacco et al. 2016). During later stages of the disease, decreases in the autophagic flux of DMD models and patients is associated with reduced regenerative capacity (Fiacco et al. 2016).

Along with the maintenance of the satellite cell capacity to regenerate, autophagy has been seen to play a role in the differentiation. During differentiation of C2C12 myoblasts and primary culture myoblasts obtained from mice, levels of autophagy increase (Fortini et al. 2016; McMillian and Quadrilatero 2014). Autophagy induction during the differentiation of C2C12 myoblasts is independent of mTORC1 activation (Fortini et al. 2016). These results indicate that the changes in autophagy activity is required for not just the viability of satellite cells but also for normal progression of skeletal progenitors into newly formed myofibers. This requirement for autophagy at various levels throughout the entire differentiation process shows its critical role for muscle regeneration.

Activating Protein-1

The activating protein-1 (AP-1) complex is formed by basic region-leucine zipper (bZIP) proteins that belong to the Jun, Fos and Maf families (Chimenov and Kerppola 2001). The proteins belonging to the Jun family (c-Jun, JunB and JunD) have the ability to form

homodimers, however members of the Fos family (c-Fos, Fra-1 and Fra2) do not have the ability to form homodimers, but form heterodimers with members of the Jun family (Shaulian and Karin 2002). AP-1 dimers composed on Jun and Fos family proteins bind to TPA cis element (12-O-tetradecanoylphorbol-13-acetate response element) with the sequence 5'- TGAC/GTCA-3' (Angel and Karin 1991). Although Fos proteins are unable to form stable homodimers, upon binding with a member of the Jun family the Fos:Jun dimers are more stable than Jun:Jun dimers (Halazonetis et al. 1988). These complexes have been observed to be involved in many cellular processes including transformation, proliferation, apoptosis and differentiation (Angel and Karin 1991). The activity of the AP-1 complex is dependent on the members of the dimer, an example of modulating AP-1 complex activity caused by dimer members can be seen in c-Jun. c-Jun has the highest activation potential of the Jun proteins (Ryseck and Bravo 1991). Upon the formation of a heterodimer with c-Fos, the transcriptional activation potential further increases due to the stabilization of the dimer (Halazonetis et al. 1988; Kouzarides and Ziff 1988). Conversely, the dimerization of c-Jun and JunB weakens the transcriptional potential of c-Jun (Chiu, Angel, and Karin 1989; Schütte et al. 1989). In human dermal fibroblast the modulation of c-Jun activity through its antagonist JunB has been seen as a critical means for the fine balance of cytokine regulation in epithelial cells (Szabowski et al. 2000). As well the reduction of c-Jun activity by JunB allows for the activation of cyclin D1 promotor in HeLa cells, allowing cells to progress in the cell cycle (Bakiri et al. 2000). Along with the modulation of AP-1 protein activity through dimerization, the proteins are also modulated through phosphorylation (Karin 1996).

Activity of AP-1 proteins is highly diverse

Although the activity of AP-1 complex is highly dependent the dimerization, AP-1 proteins have individually been associated with specific functions and activation stimuli. Jun proteins respond to many different stimuli such as UV irradiation, cytokines, oxidative stress and growth factors (Angel and Karin 1991; Angel et al. 1988). ERK/MAP kinase (MAPK) cascade causes the induction of fos genes (Karin 1996), ERKs directly phosphorylates Fra1 and Fra2 during serum stimulation (Gruda et al. 1994). Interestingly, the formation of Jun:Fos heterodimers has been shown to also lead to an increase in c-Jun gene induction through both monocyte-specific enhancer binding factor 2 (MEF2) proteins and various other transcription factors which bind to the c-Jun promoter (Han and Prywes 1995). Specifically, MEF2c activation increases c-Jun expression (Kato et al. 1997). While c-Jun activity is modulated by phosphorylation by JNK, enhancing c-Jun activity (Karin 1996).

c-Fos and FosB have been seen to participate in cell proliferation regulation. Fibroblasts which are deficient for both c-Fos and FosB were observed to have reduced proliferative capacity compared to fibroblasts deficient in with c-Fos or FosB (Brown et al. 1998). Along with c-Fos and FosB, JunD and c-Jun have been seen to play a role in proliferation. In fibroblast, a JunD double knockout in mice correlated with reductions in both fibroblast proliferation as well as decreases in fibroblast cell size (Han et al. 1997). Knockdowns of c-Jun in fibroblasts have been seen to lead to defects in fibroblasts and are unable to maintain prolonged quiescence (Johnson et al. 1993). In fibroblasts which have been subjected to UV-irradiation, constant c-Jun expression leads to cells which

lack cell cycle control – cells that are unable to stop and continue the cell cycle (Shaulian et al. 2000). As well as proliferation, AP-1 proteins play a role in apoptosis. Much like the varying roles AP-1 proteins take in proliferation, AP-1 proteins can both be proapoptotic and anti-apoptotic depending on both AP-1 protein and cellular conditions. Increased levels of c-Jun activity in neuronal cells induces apoptosis in vitro, through the regulation of the critical pro-apoptotic Bcl2 family member Bcl-2-like protein 11 (Bim) (Ham et al. 1995).

AP-1 in Skeletal Muscle Differentiation

Along with AP-1's role in proliferation and apoptosis, a variety of the proteins play critical roles in myogenic cell differentiation. Initial investigations of c-Jun in myogenic cells suggested c-Jun inhibited autophagy by dimerizing with MyoD leading to its inhibition (Bengal et al. 1992). During the differentiation of C2C12 myoblasts, AP-1 proteins c-Jun, JunD and Fra2 were observed during the entirety of the differentiation of myoblasts into myotubes (Andreucci et al. 2002). Two distinct AP-1 forms of complexes were observed during the course of differentiation; complexes of either c-Jun:Fra-2 or JunD:Fra-2 heterodimers in differentiating (Andreucci et al. 2002). c-Fos was seen as a candidate for the heterodimer partner for AP-1 complexes in C2C12 myoblast in the proliferative state due to the high level of c-Fos observed in previous studies (Andreucci et al. 2002). c-Fos downregulation by MyoD is required for myogenic cells to progress into differentiation (Trouche et al. 1993). Growth factor signaling decreases as cells are initiated into differentiation c-Jun expression is seen throughout, which is of particular interest as c-Jun transcription was previously observed to be dependent on growth

factor signaling (Andreucci et al. 2002). The maintained expression of c-Jun is attributed to MEF2 activity in HeLa cells (Andreucci et al. 2002). These AP-1 complexes were seen positively correlate with myogenetic differentiation. Fra-2 has also been observed to inhibit differentiation when stabilized through phosphorylation by ERK 1/2 (Alli et al. 2013). In C2C12 reserve cells the loss of Fra-2 expression through siRNA knockdowns leads to an increase in myogenic differentiation markers MCK and Myosin Heavy Chain (MyHC) (Alli et al. 2013). Fra-2 was observed to be present in C2C12 reserve cells as well as mouse isolated satellite cells on myofibers, while being reduced in C2C12 myotubes (Alli et al. 2013). These findings suggested that Fra-2 played an inhibitory role in myogenic differentiation in non-differentiating myogenic cells maintaining a quiescent state (Alli et al. 2013). The various roles of AP-1 proteins indicated that AP-1 proteins have various effects dependent on both the tissue context, as well as the dimerization which results in the complex.

Relationship Between AP-1 and Autophagy

An emerging role for AP-1 complexes has been the regulation of autophagy. AP-1 Jun proteins have been investigated in their role of autophagy in cancer cell lines. Jun proteins c-Jun and JunB have been shown to be downregulated during nutrient starvation induced autophagy in both immortalized and embryogenic derived fibroblasts (Yogev et al. 2010). The inactivation of mTOR by Rapamycin positively correlated with a decreased level of JunB in HEK293 cells (Yogev et al. 2010). The regulation of JunB at the post-transcriptional level occurs in mTOR inhibition autophagy induction through Rapamycin treatment, as well as nutrient starvation induced autophagy in HEK293 cells

(Yogev et al. 2010). Along with JunB, c-Jun also had an inhibitor effect on nutrient starvation induced autophagy in HEK293 cells (Yogev et al. 2010). The inhibitor effect of c-Jun on starved HEK293 cells also appears to be JNK activation independent, as c-Jun with mutated JNK phosphorylation sites inhibit autophagy similar to the wildtype c-Jun (Yogev et al. 2010). The non-essentiality of c-Jun phosphorylation indicates that the involvement of c-Jun can occur without further activity enhancement through JNK, alluding to another pathway of activation for autophagy inhibition. However, due to the unique nature of AP-1 proteins, and the various roles they can take dependent of context, c-Jun has also been associated with the induction of ceramide-mediated autophagy in Human Epidermoid carcinoma 3 (Hep3B) cells (Li et al. 2009). In response to ceramide treatment, Hep3B cells showed increased levels of JNK activation and c-Jun activation which positively correlated with an increase in Beclin-1 and LC3 I/II (Li et al. 2009). The correlated increase was further explored in sequence analysis revealing a c-Jun binding site within the Beclin-1 promoter, and an increase in c-Jun binding to the Beclin-1 promoter region with ceramide treatment in Hep3 cells (Li et al. 2009). AP-1 protein c-Jun has also been shown to regulate the transcription of LC3 in autophagy during ceramide treatment of human nasal carcinoma (CNE2) cells (Sun et al. 2011). These results indicate that in ceramide induced autophagy in tumor cells, JNK signaling leading to the phosphorylation of c-Jun directly promotes increases in transcription of Beclin-1 as well as LC3, increasing autophagy. AP-1 proteins appear to play both positive and negative roles in autophagy. The role of AP-1 appears to be both dependent on the autophagy induction agent, and its dimer pair.

Statement of Purpose

Previous studies have shown that AP-1 proteins play a role in various stages of the differentiation of skeletal muscle. The composition of AP-1 complexes change throughout differentiation (Andreucci et al. 2002). As well, c-Jun has been observed to interact with MyoD to inhibit differentiation (Bengal et al. 1992). Fra-2 has also been observed to inhibit differentiation in C2C12 myoblasts in both C2C12 reserve cells and SCs (Alli et al. 2013).

Recent literature has shown that autophagy is a key regulator in the maintenance of quiescence in SCs (García-Prat et al. 2016). Autophagy activity has also been seen to change throughout stages of differentiation and regulate differentiation in both mouse derived primary myoblasts and C2C12 myoblasts (Fortini et al. 2016; McMillan and Quadrilatero 2014).

AP-1 proteins have been implicated in both the promotion and inhibition of autophagy in various cancer cell types. Thus, AP-1 proteins may a role in the regulation of autophagy in skeletal muscle progenitor cells. To assess the role of AP-1 in autophagy in skeletal muscle progenitor cells, this study examined the effects of AP-1 proteins in both basal and induced autophagy conditions. It is hypothesized that AP-1 proteins will regulate autophagy in skeletal muscle progenitor cells. AP-1 proteins are downstream targets of known autophagy regulation pathways (Figure 3).

Understanding the mechanisms that regulate autophagy in skeletal muscle progenitor cells aids in understanding the maintenance of quiescence in SCs and the preservation of injury response capabilities in aged individuals and disease models.

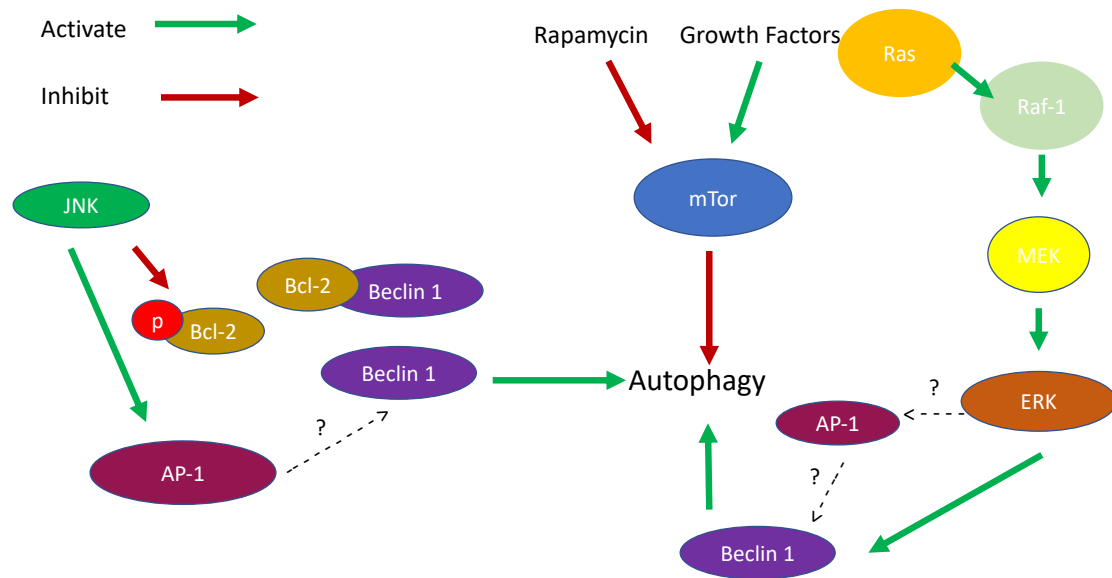


Figure 3: Proposed relation of AP-1 proteins to autophagy.

A schematic of autophagy induction pathways. Autophagy can be inhibited by mTOR in its activated state. Rapamycin inhibits mTOR leading to the induction of autophagy. Autophagy can also be induced through Beclin-1- a key component in the autophagy induction complex. Beclin-1 is inhibited by Bcl-2 and can be indirectly activated by JNK through the phosphorylation of Bcl-2 leading to its dissociation from Beclin-1. Ras also acts through its downstream target ERK to regulate Beclin-1. AP-1 proteins are downstream targets of ERK or JNK pathways which may contribute to upregulation or downregulation expression of autophagy critical components such as Beclin-1.

Introduction

Major motor movements, including walking, running, grasping and the ability to maintain upright posture is dependent on skeletal muscles. Mature skeletal muscle is the result of myogenesis, the process in which skeletal muscle progenitor cells undergo differentiation to mature into muscle fibers. During the process of myogenesis, a distinct cohort of myoblasts, SCs, continuously express Pax7 and maintain a quiescent state. SCs are critical progenitors for post-natal muscle injury repair and regeneration (Lepper, Partridge, and Fan 2011). Upon injury, SCs produce myoblasts that further differentiate to form new muscle fibers as well as additional SCs to maintain a pool of quiescent cells for future injury events (Bentzinger, Wang, and Rudnicki 2012; Buckingham et al. 2003; Zammit 2006). SCs have elevated levels of autophagy, which has been demonstrated to be critical for the SC's ability to maintain quiescence (García-Prat et al. 2016). Declines in autophagy lead to SCs entering senescence and have been shown in both DMD and aged individuals (Fiacco et al. 2016; García-Prat et al. 2016). Decline in quiescent SCs are associated with declines in regenerative capacity in the individual. Autophagy activity itself changes through the entirety of differentiation, and the changes in autophagy are critical for cells' progression into differentiation (Fortini et al. 2016). Autophagy's critical role in the entire process of muscle differentiation and its requirement for maintenance of quiescence of SCs makes it a key component in skeletal muscle regeneration.

Autophagy is a highly conserved cellular process. Autophagy maintains homeostasis both in basal conditions and during times of stress; it can be induced by many stimuli (Mehrpour et al. 2010). AP-1 proteins are the downstream targets of mTOR

independent autophagy pathways. Both JNK and ERK 1/2 are upstream regulators of autophagy and have been seen to induce autophagy in cancer cells in various growth conditions and in response to therapeutic agents (Sui et al. 2013).

AP-1 protein c-Jun is a downstream target of JNK. Downstream phosphorylation of JNK, c-Jun, has been detected to both inhibit and promote autophagy in cancer cells. In the promotion of autophagy, c-Jun has been observed to bind to Beclin-1 – a critical autophagy initiation complex protein- promoter region under ceramide treatment in cancer cells (Li et al. 2009). However, in cases of nutrient starvation in cancer cells c-Jun inhibits starvation induced autophagy (Yogev et al. 2010). In the case on nutrient starvation, JunB also suppressed autophagy (Yogev et al. 2010).

The highly conserved nature of autophagy as well as upstream regulators of AP-1 proteins have already established roles in cancer cells autophagy regulation, suggesting a possible role for AP-1 in autophagy in muscle progenitor cells. AP-1 proteins may a role in the regulation of autophagy in skeletal muscle progenitor cells in both basal and induced autophagy conditions. In this study we show that AP-1 proteins can act to both enhance and suppress autophagy in muscle progenitor cells. We show that the effect of Jun proteins is dependent on the particular growth conditions of the cells.

Material and Methods

Cell Culture

C2C12 myoblasts were purchased from American Type Culture. Cells were maintained in growth media composed of Dulbecco's modified Eagle's medium (DMEM) with high glucose and L-glutamine (HyClone) supplemented with 10% foetal bovine serum (HyClone) and 1% penicillin/streptomycin (Invitrogen). Cells were maintained in a humidified 37 °C incubator at 5% CO₂ and replenished with fresh medium every 48 hours.

Detailed isolation protocol can be found in Appendix. Briefly, primary cultures of skeletal muscle were obtained from CD-1 neonatal mice (1-3 days old) using methods described by Hindi (2017). Following pre-plating selections as described by Hindi (2017), cells were maintained in primary culture growth media containing F-10 media with 20% foetal bovine serum (HyClone), 1% penicillin/streptomycin (Invitrogen) and 10ng/mL basic fibroblast growth factor (bFGF). Cells were maintained in a humidified 37 °C incubator at 5% CO₂ and replenished with fresh medium every 48 hours.

Autophagy induction in C2C12 cells

Detailed protocol can be found in Appendix A. Autophagy induction was accomplished using a treatment of 10µg/mL of Rapamycin (sc-3504, Santa Cruz Biotechnology) added to C2C12 growth media for 6 hours, or through 6 hour serum starvation in which cells were cultured in DMEM with 1% penicillin/streptomycin.

Transfection

Detailed protocol can be found in Appendix A, briefly, C2C12 myoblasts were transfected using Polyethyleneimine (PEI). Cells were transfected using a 1:3 DNA (μg) to PEI (μg) ratio in C2C12 growth media. A total of $3\mu\text{g}$ of DNA was used for 6 well plates with $9\mu\text{g}$ PEI. Cells were harvested 48 hours post transfection.

siRNA transfection of C2C12 Myoblasts

siRNA transfections were performed using calcium precipitation method. Detailed protocol can be found in Appendix A. c-Jun siRNAs were used at a concentration of 100nM. Cells incubated with siRNA calcium precipitation mixture overnight in DMEM. Cells were then refreshed with C2C12 growth media. Cells were then harvested 48 hours post- transfection.

siRNA transfection of Primary Culture

siRNA transfections were performed using Lipofectamine 2000, siRNAs were used at a concentration of 100nM. Detailed protocol can be found in Appendix A. Briefly, prior to transfection, cells were refreshed with Primary culture growth media. For a 6-well plate, siRNAs were mixed with $4\mu\text{g}$ Lipofectamine 2000 in Opi-MEM media. Cells were then incubated in $500\mu\text{L}$ Opi-MEM and siRNA, Lipofectamine for 4 hours. Primary culture growth media was then added to the cells. Cells were harvested 48 hours post transfection.

Antibodies and reagents

The following antibodies were purchased from Santa Cruz Biotechnology: actin (sc-1616), c-Jun H-79 (sc 1694), JunB, JunD, MyoD. Anti-LC3B (2775), anti-c-Jun phosphor-Ser 63 (54B3) were purchased from Cell Signalling. The following siRNAs were purchased from Sigma-Aldrich: c-Jun si356 (SASI_Mm01_00046356), c-Jun si357 (SASI_Mm01_00046357), c-Jun si894 (SASI_Mm02_00318894_AS), Scrambled (SIC001).

Immunoblots

Cells were washed with 1x PBS and lysed in NP-40 lysis buffer (50mM Tris, 150 mM NaCl, 0.5% NP-40, 2mM EDTA, 100 mM NaF and 10 mM Na pyrophosphate) containing protease inhibitor cocktail (Sigma- Aldrich), 1mM phenylmethylsulfonyl fluoride (Sigma- Aldrich) and 1 mM sodium orthovanadate (Bioshop). Protein concentrations were determined by Bradford assay (Bio-Rad). Twenty micrograms of total protein were resolved to 10% SDS-PAGE and then transferred onto Immobilon-P membranes (Millipore) for 1 hour or overnight. Non-specific binding sites were blocked using 5% milk in PBS. Membranes were incubated with primary antibodies overnight at 4°C in 5% milk in PBS or 5% BSA in TBST. Horseradish peroxidase-conjugated secondary antibody was added for 2h at room temperature. Protein was detected with ECL chemiluminescence reagent (Pierce).

Fluorescence Microscopy of Tandem tagged- LC3 positive C2C12 Myoblasts

Cells were transfected with 1.5ug tandem tagged LC3 plasmid (tLC3) along with 1.5ug of c-Jun DNA or control DNA using PEI method. C2C12 myoblasts were fixed using 4% paraformaldehyde at room temperature for 5 minutes 48 hours post-transfection.

Images were acquired using a Zeiss Observer Z1 microscope.

Primary Skeletal Muscle Fiber Isolation and Immunofluorescence Analysis

Myofibers were isolated from the muscle -extensor digitorum longus (EDL)- of 6-8 week-old male C57B1/6 mice as previously described by Alli et al. (2013), a detailed protocol is described in Appendix. Fibers were fixed in 4% paraformaldehyde in PBS for 5 minutes and washed three times with PBS. Myofibers were blocked using 10% Horse Serum in PBS for 1 hour. Blocking solution was changed, and primary antibodies were added to fresh blocking solution. Primary antibodies used were: monoclonal mouse anti-Pax7 (Developmental Studies Hybridoma Bank) 1:2, c-Jun, JunB (Santa Cruz) 1:500. The primary antibodies were incubated overnight with fibers at 4°C. Fibers were then washed in PBS three times and incubated with secondary antibodies. Secondary antibodies used: (1:1000) dilution for 2 hours at room temperature. Nuclei were visualized using DAPI counterstain incubation for 30 minutes (1:1000). Myofibers were mounted in Fluorescent mounting medium (DAKO cytomation, Cambridgeshire, UK). Mounted myofibers were imaged using Zeiss Observer Z1 microscope.

Animal Care

Animals were cared for in accordance with the Institutional Animal Care and Use Committee of York University.

For single fiber experiments, 6- to 8- week old male mice were obtained from Charles River. Mice were sacrificed using cervical dislocation in accordance with the Institutional Animal Care and Use Committee of York University.

For primary culture experiments, neonatal mice (1-3 days old) were sacrificed using decapitation in accordance with the Institutional Animal Care and Use Committee of York University.

Immunofluorescence of Primary Culture

Primary cultures of skeletal muscle were fixed using 4% paraformaldehyde at room temperature for 5 minutes. Cells were permeabilized with 90% methanol in -20 °C for 6 minutes. Following permeabilization cells were blocked using 10% goat serum in PBS for 30 minutes at room temperature. 0.1% BSA in TBS was then added to cells along with primary antibodies. . Primary antibodies used were: monoclonal mouse anti-Pax7 (Developmental Studies Hybridoma Bank) 1:2, MyoD (Santa Cruz 1:500). Secondary antibodies used: FITC and TRITC (1:1000) dilution for 2 hours at room temperature. Nuclei were visualized using DAPI counterstain incubation for 30 minutes (1:1000). Cells were Images were acquired using a Zeiss Observer Z1 microscope.

Statistical Analysis

Statistical analysis for siRNA knockdown experiments in both C2C12 myoblasts and skeletal muscle primary culture conditions was carried out using a non-parametric ANOVA Kruskal-Wallis test. The Dunn's multiple comparisons test was applied with comparisons between siRNA groups and control. Statistical analysis for ectopic expression of c-Jun in C2C12 myoblasts with autophagy induction was carried out using Mann-Whitney test to compare within groups. Statistical analysis of tLC3 puncta in C2C12 myoblasts was carried out using Mann-Whitney test. Non-parametric tests were used as data sets failed Anderson-Darling normality test for normal distribution.

Results

c-Jun is expressed in satellite cells

Immunostaining for c-Jun was performed on individual skeletal muscle fibers (Figure 4). It has been previously shown that AP-1 protein Fra-2 was present in satellite cells (Allie et al. 2013). It was speculated that c-Jun may be visualized in satellite cells as c-Jun is a known heterodimer partner to Fra-2 and, shown through protein assays is present in myoblasts (Allie et al. 2013). c-Jun was found expressed in the Pax7 positive cells following isolation. c-Jun localization was restricted to Pax7 positive cells and did not occur in myofibers nuclei. These c-Jun and Pax7 positive cells were observed on the outer regions of the myofibers, consistent with the localization of satellite cells. Both the co-localization of the Pax7 and c-Jun along with location of the positively stained cells supported our idea that c-Jun is present in satellite cells.

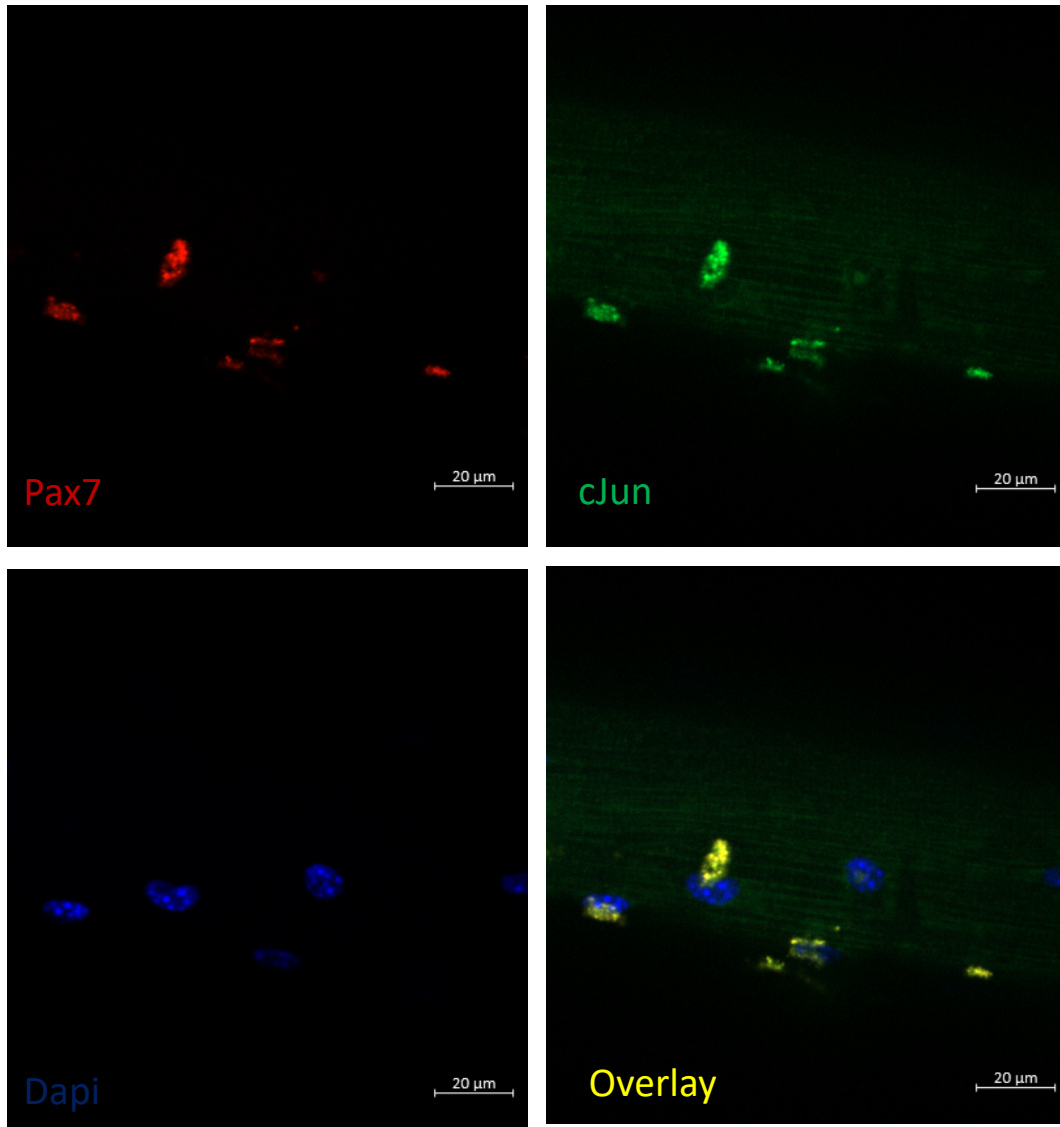


Figure 4: c-Jun localization in satellite cells on myofiber.

Representative image of a single myofiber was dissected from EDL muscle of 6-8-week-old mice. Fibers were immunostained for c-Jun (green) and Pax7 (red) following fixation. Nuclei were stained with DAPI (blue). Two biological replicate experiments were conducted.

Endogenous c-Jun reduces LC3II autophagosome content in proliferative conditions in C2C12 myoblasts

To observe the effect of endogenous c-Jun on proliferating myoblasts, c-Jun expression was reduced using 3 different siRNA in mouse myoblast C2C12 cells. C2C12 myoblasts were selected as our experimental model as they express myogenic markers similar to human skeletal muscle. C2C12 myoblasts cells also progress into differentiation much like human skeletal muscle and in proliferative growth conditions provided a myogenic progenitor stable cell line. In the proliferative state, C2C12 myoblasts have characteristics of myocytes.

The cell lysate was subjected to a Western immunoblot. A reduction of c-Jun accomplished using three different siRNAs (Figure 5). As shown in Figure 4 the reduction of c-Jun led to an increase in overall LC3. To access the changes in autophagosome content, we observed the change in the amount of LC3 II in each condition. This measurement allowed for the observation of autophagosome content, however does not allow to conclusions on changes in autophagic flux. This measurement indicates a change occurs in autophagy however conclusion cannot be drawn on if this change is an increase or decrease of autophagy or flux. A significant increase was noted in LC3 II when compared to controls in one of the three siRNA knockdown conditions (Figure 5). Results show that the suppression of endogenous c-Jun in proliferative conditions caused an increase LC3II content in autophagosomes of C2C12 cells.

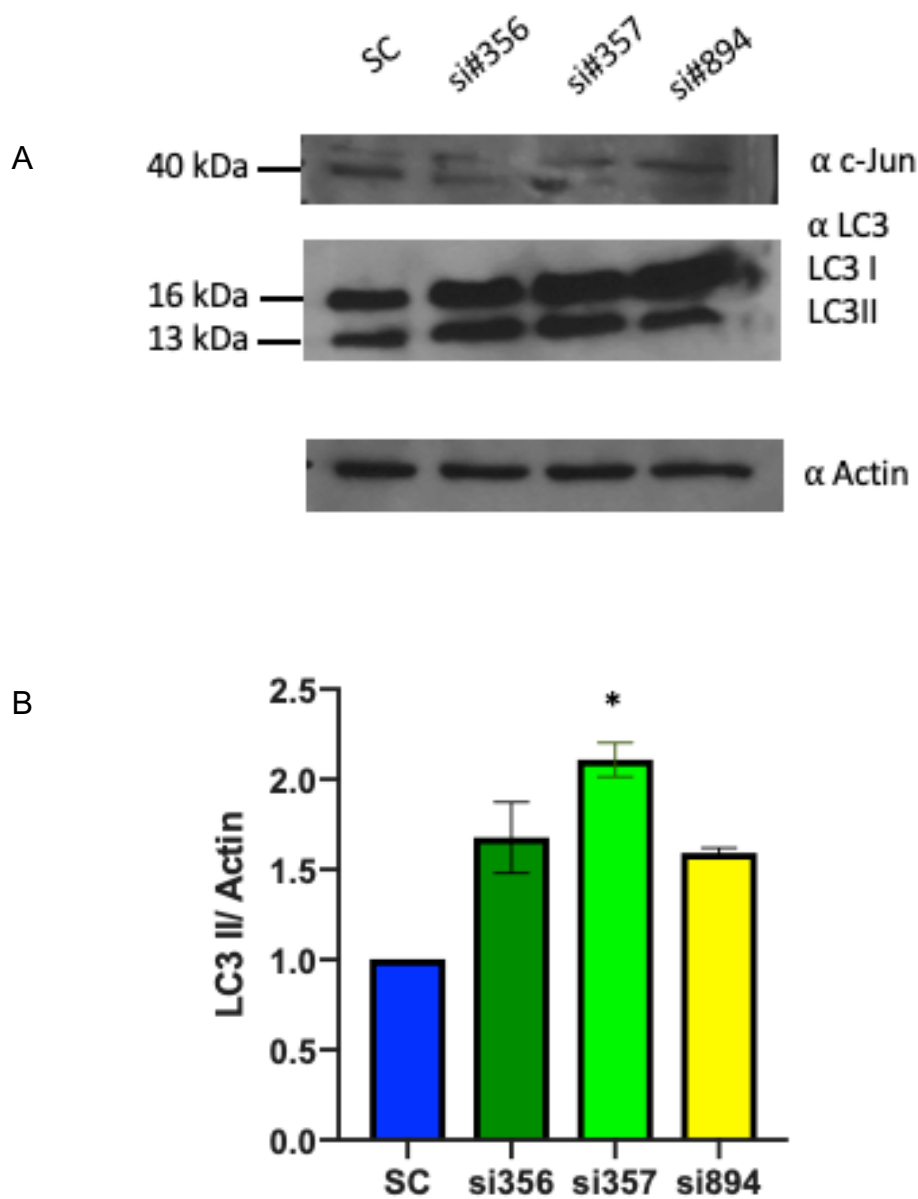


Figure 5: Reduction of endogenous c-Jun leads to an increase in LC3II in C2C12 myoblasts.

(A) siRNA-mediated knockdown of c-Jun in C2C12 cells. C2C12 cells were transfected with three different siRNAs and harvested 48 hours post-transfection for western blot analysis. SC indicates scrambled siRNA control.

(B) Densitometric values of LC3 II expression in C2C12 cells following siRNA-mediated knockdown of c-Jun relative to control. Values normalized to loading control. Statistical analysis using a non-parametric Dunn's multiple comparison test; * $p = 0.0412$. Values quantified using ImageJ (n=2 two biological replicates, mean \pm s.e.m).

Reduction of endogenous c-Jun in primary skeletal muscle culture reduces LC3II autophagosome content

To assess the effects of c-Jun on autophagy in primary skeletal muscle cultures c-Jun expression was reduced using two different siRNAs which had previously been successful in c-Jun knockdown in C2C12 myoblasts (Figure 6). Primary skeletal muscle cultures were obtained from CD-1 mouse hindlimb muscles. These cells are unique from C2C12 cells as they are in a proliferative state and are myoblasts, a less mature state of myogenic progenitors compared to C2C12 cells in proliferative state which are characteristically myocytes. Isolated primary cultures contained myogenetic progenitor cells, myoblasts positive for both MyoD and Pax7, as well as myoblasts positive for MyoD and lacked Pax7 expression (Figure 6a). Cells positive with only Pax7 were not detected. Unlike C2C12 myoblasts, the knockdown of c-Jun lead to a decrease in autophagy in primary culture. A reduction was found in overall LC3 expression, as well as a decrease in the LC3 II form relative to control (Figure 6b).

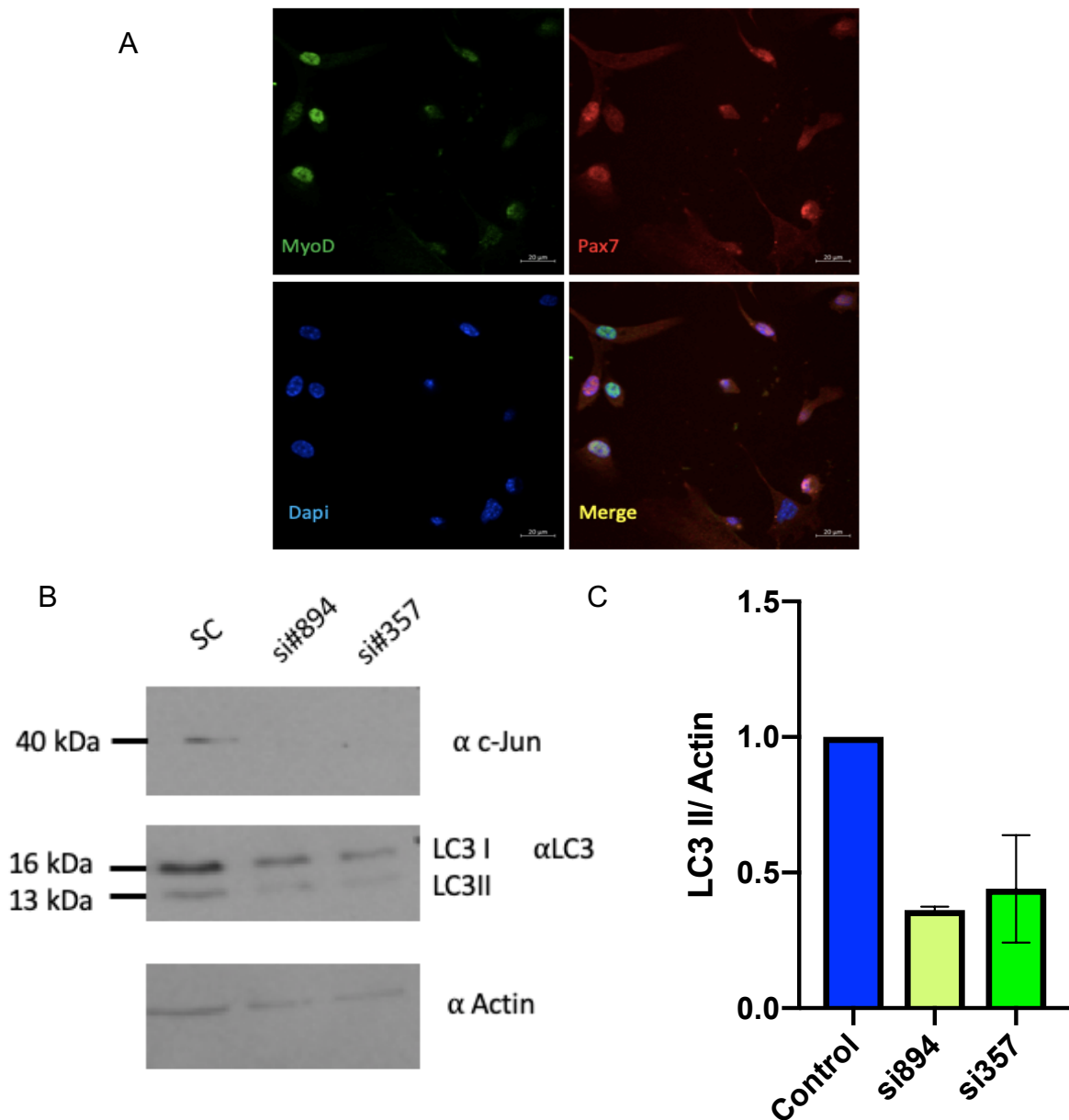


Figure 6: Reduction of c-Jun in primary skeletal muscle culture decreases LC3II autophagosome content.

(A) Representative image of immunofluorescence of primary culture from neonatal mice. Cells were fixed and stained for MyoD (green) and Pax7 (red). Nuclei were stained using DAPI. **(B)** Representative blot of siRNA-mediated knockdown of c-Jun in C2C12 cells. C2C12 cells were transfected with two different siRNAs and harvested 48 hours post-transfection for western blot analysis. SC indicates scrambled siRNA control. **(C)** Densitometric values of LC3 II expression in primary culture following siRNA-mediated knockdown of c-Jun relative to control. Values normalized to loading control. . Statistical analysis using a non-parametric Dunn's multiple comparison test. Values quantified using ImageJ ($n=2$ biological replicates, mean \pm s.e.m).

Reduction of endogenous c-Jun in C2C12 myoblast has no effect on Rapamycin induced autophagy

The opposition effects of c-Jun in primary skeletal muscle cultures and C2C12 myoblasts alluded to c-Jun having a specific effect dependent on cell type as well as possibly autophagy induction mechanism. The specific effects of c-Jun on autophagy have been previously shown in the literature in various cancer cell lines. In order to further investigate the apparent specificity of c-Jun on autophagosome content in skeletal muscle progenitors, c-Jun expression was reduced in C2C12 myoblast using siRNAs while inducing autophagy through Rapamycin treatment (Figure 7).

The reduction of endogenous c-Jun through siRNA knockdown combined with autophagy induction due to Rapamycin treatment led to no observable change in the observed amount of LC3 II compared to control in observed autophagy in C2C12 myoblasts. The reduction of c-Jun expression had no apparent effect on Rapamycin induced autophagy in C2C12 myoblast when observing changes in autophagosome content.

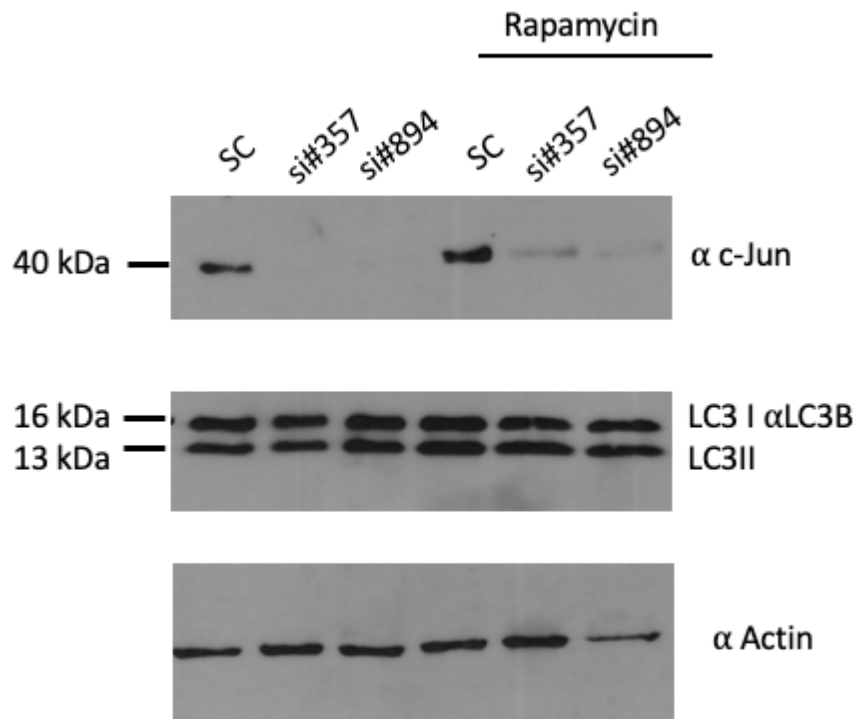


Figure 7: Reduction of c-Jun has no effect on Rapamycin induced autophagy autophagosome content.

Representative immunoblot of siRNA knockdown of c-Jun in C2C12 myoblasts. Cells then were grown in growth media (GM) or treated with Rapamycin (10 μ g/ml) for 6 hours prior to harvest. C2C12 myoblasts were harvested 48 hours post-transfection for western blot analysis. Two biological replicates were preformed

Ectopic expression of c-Jun has unique effects on autophagosome content dependent on mode of autophagy induction

A reduction in endogenous c-Jun led to an increase in total LC3 and in LC3 II in C2C12 cells in basal growth conditions (Figure 5) and had no effect on rapamycin induced autophagy (Figure 7). To further evaluate the possibility c-Jun effecting autophagosome content in skeletal muscle progenitors, Ectopic expression of c-Jun was induced in C2C12 cells to assess its effect on both basal and induced autophagy.

To observe the effect of ectopic expression of c-Jun on autophagosome content c-Jun was overexpressed in growth conditions as well as in two autophagy induction conditions, Rapamycin treatment and Serum-Free conditions (Figure 8). Rapamycin was used to specifically inhibit mTORC, which leads to the induction of autophagy, while serum withdrawal has been shown to induced autophagy (Klionsky et al. 2012). Interestingly the ectopic expression of c-Jun in C2C12 myoblasts in growth conditions led to an increase in LC3II autophagosome content, shown by a significant increase of GFP⁺RFP⁺ and RFP⁺ puncta, and an increase in LC3 II when observed by immunoblot- although this result was not significant. When C2C12 myoblasts were treated with Rapamycin, there was an increase in c-Jun and JNK activity observed through the JNK phosphorylation site Ser63 on c-Jun (Figure 8a). The ectopic expression of c-Jun coupled with Rapamycin induced autophagy led to a decrease in LC3II autophagosome content relative to the Rapamycin induced state (Figure 8b) . The reduction of LC3II content due to c-Jun in instances of induced autophagy seems to specific on condition; Serum-Free induction of autophagy was un-effected by ectopic expression of c-Jun.

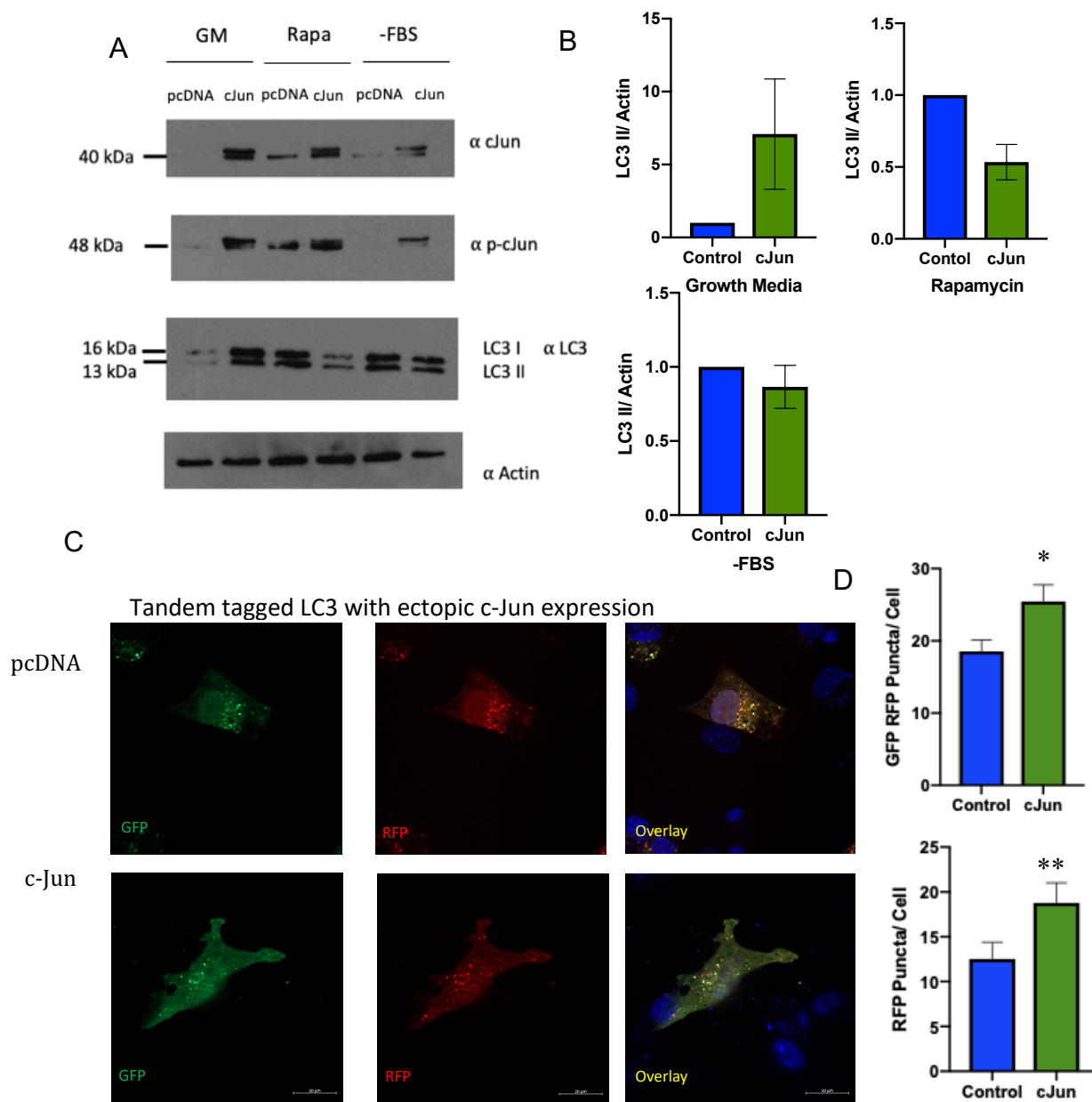


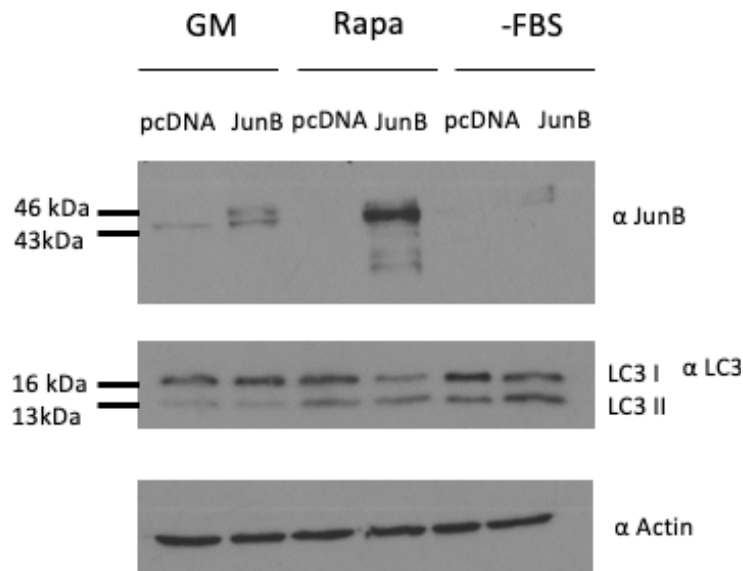
Figure 8: Ectopic expression of c-Jun regulates autophagosome content dependent on autophagy induction method in C2C12 myoblasts.

(A) Representative blot of ectopic expression of c-Jun in C2C12 myoblasts. Cells then were grown in growth media (GM), treated with Rapamycin (10 μ g/ml) for 6 hours (Rapa), or treated with serum free media for 6 hours (-FBS) prior to harvest. C2C12 myoblasts were harvested 48 hours post-transfection for western blot analysis. (B) Densitometric value of LC3 II expression in C2C12 cells following ectopic expression of c-Jun and treatments. Values quantified using ImageJ (n=2 biological replicates, mean \pm s.e.m). (C) Representative images of ectopic expression of c-Jun in tandem tagged LC3 expressing C2C12 cells to monitor autophagy in C2C12 myoblasts in GM. (D) Averages of both GFP RFP positive puncta and RFP puncta. Statistical analysis carried out using Mann-Whitney * $p=0.0158$, ** $p=0.025$ (A total of 50 cells per condition were counted from 2 biological replicates, mean \pm s.e.m)

AP-1 Proteins have unique effects on induced autophagy in C2C12 myoblasts

AP-1 proteins previously been shown to have unique characteristics on autophagy dependent on factors such as cell type and autophagy induction mechanism. Because of AP-1 proteins previously described unique natures, Jun proteins were investigated in their effects on autophagy in C2C12 cells. Initial investigations focused on JunB, a previously documented repressor of autophagy in (Yogev et al. 2010), as well as JunD. The ectopic expression of JunB was successfully achieved in C2C12 myoblasts as observed in Figure 9a. The ectopic expression of JunD was not achieved (Figure 9b). JunB and JunD were ectopically expressed in C2C12 myoblasts and autophagy was induced using both Rapamycin and serum withdrawal. Figure 9 shows that unlike c-Jun (Figure 8a), the ectopic expression in growth conditions led to no changes in autophagosome content. However, JunB appeared to have a reductive effect on Rapamycin induced LC3II autophagosome content in myoblasts (Figure 9). Figure 8 also shows that unlike c-Jun, neither JunB or JunD expression were increased with Rapamycin treatment.

A



B

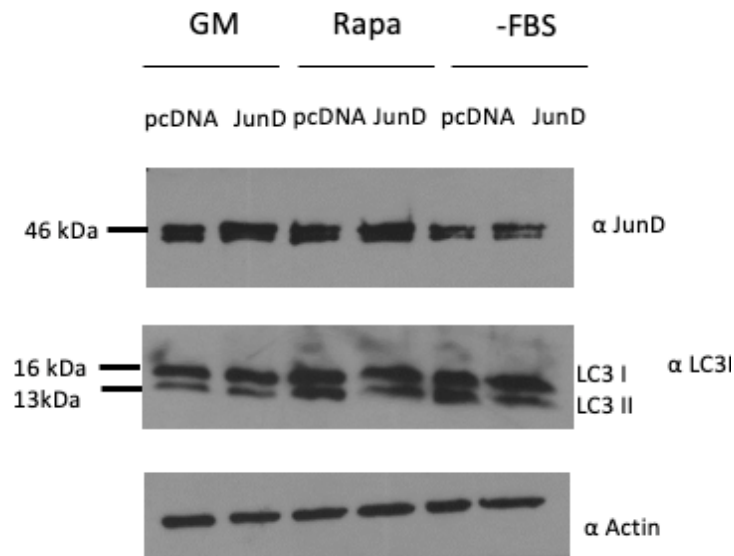


Figure 9: Jun proteins reduce induced autophagy. (A) Ectopic expression of JunB (A) and JunD (B) in C2C12 myoblasts. Cells then were grown in growth media (GM), treated with Rapamycin (10 μ g/ml) for 6 hours (Rapa), or treated with serum free media for 6 hours (-FBS) prior to harvest. C2C12 myoblasts were harvested 48 hours post-transfection for western blot analysis. Immunoblot represents single measurement.

Discussion

It has been shown that composition of AP-1 complexes changes throughout differentiation, suggesting specific AP-1 components play a role in the regulation of differentiation (Andreucci et al. 2002). In particular, c-Jun has been shown to inhibit MyoD leading to an inhibition of differentiation (Bengal et al. 1992). As well, AP-1 protein Fra-2 specifically has been observed to be present in SCs and has been shown to inhibit differentiation of myoblasts (Alli et al. 2013). Both c-Jun and Fra2 have been observed in myogenic progenitor cells (Alli et al. 2013). AP-1 proteins c-Jun and JunB have previously been implicated in the regulation of autophagy in cancer cells (Pattingre et al. 2009; Yogev et al. 2010). However, the effect of AP-1 proteins appears to be dependent on the method which autophagy is induced as well as cell type (Pattingre et al. 2009; Yogev et al. 2010).

The focus of this study was to examine the regulation of autophagy in skeletal muscle progenitor cells by AP-1 proteins. AP-1 proteins are downstream targets of ERK and JNK, both which are known upstream regulators of autophagy, and have been of interest in autophagy regulation in cancer.

Recent studies have shown that autophagy is essential for the preservation of quiescence in satellite cells and a reduction of autophagy is associated with both age and regenerative capacity of skeletal muscle (García-Prat et al. 2016). Autophagy as also been shown to be altered in muscle wasting disease DMD. In the early stages of DMD, tibialis anterior muscles appear to have greater levels of autophagic flux in comparison to non- disease controls, however in later stages autophagic flux decreases are observed, becoming equivalent to or less than control levels (Fiacco et al. 2016).

The change in autophagy levels corresponds to the muscle wasting severity of the disease progression- the early stages autophagy appear to contribute to regenerative compensation in DMD (Fiacco et al. 2016). Autophagy contributes to the skeletal differentiation process and has been implicated as critical for fusion and differentiation (Fortini et al. 2016; McMillan and Quadrilatero 2014).

As there has been continued exploration of the role of autophagy in various aspects of skeletal muscle differentiation and the quiescence of satellite cells, this study aimed to explore the regulation of this important process in skeletal muscle progenitor cells.

This study has shown that c-Jun is present in Pax7 positive satellite cells (Figure 4).

These results combined with previous findings that c-Jun is expressed in myoblasts (Alli et al. 2013), suggest that c-Jun may be important in both processes specific to myoblasts and satellite cells. In C2C12 myoblasts, the suppression of endogenous c-Jun led to an increase of LC3II content in autophagosomes (Figure 5). However, reductions of c-Jun in primary culture containing a heterogenous population of myogenic progenitors—both Pax7 positive and negative cells— led to a reduction of LC3II content in autophagosomes (Figure 6b). The opposing results seen in the reduction of c-Jun in C2C12 compared to primary culture conditions could be the result of the cell population in the primary conditions. C2C12 myoblasts do not express Pax7 and are fully committed showing characteristics of myocytes pre-fusion state. . Primary culture as shown in Figure 5a containing a mixed population of committed myoblasts which are Pax7⁻/MyoD⁺ and Pax7⁺/MyoD⁺ cells which can be said to be less mature and have the characteristic of satellite cells and could potentially have different AP-1 dimer complexes. The difference in the basal autophagy functions of myoblasts and satellite

cells has been shown in the literature; satellite cells typically have higher autophagy, compared to committed myoblasts, and a decline in autophagy is required for initial steps of differentiation (Erbay and Chen 2001; Fiacco et al. 2016; McMillian and Quadrilatero 2014). As well, the effects of Jun proteins are often highly dependent to context, therefore the opposing effects seen in C2C12 cells and primary culture could be due to both the differences in basal autophagy levels as well as the distinct cell characteristics. Interestingly the ectopic expression of c-Jun in C2C12 myoblasts also leads to an increase of LC3II content in autophagosomes in growth conditions (Figures 8a,c,d). Overall these results indicate that disruption to c-Jun expression leads to changes in basal autophagosome content in both C2C12 myoblasts and heterogenous populations of myogenic progenitors.

The ectopic expression of c-Jun in C2C12 myoblasts increased LC3II in autophagosome content in growth conditions. This result was contrary to what was expected to see, as both a suppression of endogenous c-Jun and ectopic expression of c-Jun yielded the same increase of LC3II autophagosome content. The reduction and ectopic expression of c-Jun suggests that c-Jun itself may have both a repressive and enhancing role in C2C12 myoblasts. The non-linear relationship seen between c-Jun expression and autophagy in basal conditions alludes to c-Jun possibly contributing to the overall autophagy homeostasis in growth conditions.

The ectopic expression of c-Jun repressed Rapamycin induced autophagy in C2C12 myoblasts (Figure 8 a, b). Upon the suppression of endogenous c-Jun in C2C12, Rapamycin induced autophagy in myoblasts experienced no alteration in autophagosome content (Figure 7). These data suggest that the effect of c-Jun on

autophagosome content in myoblasts is dependent on the induction pathway of autophagy. The results seen in myoblasts are similar to what has been observed in autophagy studies in cancer cell lines (Li et al. 2009; Sui et al. 2013; Yogev et al. 2010). Starvation induced autophagy was repressed in HEK293 cells by ectopic expression of c-Jun (Yogev et al. 2010). However, in the induction of autophagy by ceramide in Hep3b and CNE2 cells, c-Jun is critical for the expression of Beclin-1 and an increase in autophagy (Li et al. 2009). These data in conjunction with our results suggest that the effect of c-Jun on autophagosome content in myoblasts is dependent on the induction pathway of autophagy. We also observed an increase in c-Jun during Rapamycin treatment (Figure 8a).

An increase of c-Jun and c-Jun phosphorylation due to Rapamycin treatment has been observed in human rhabdomyosarcoma (Rh30 cells) (Huang et al. 2003). Huang and colleagues (2003) detected the effects of JNK activation and Rapamycin induced apoptosis. Although Huang et al.'s (2003) study does not explicitly observe autophagy, its observations of mTOR activity in conjunction with c-Jun activation show an mTOR regulation pathway of c-Jun in Rh30 cells. The increase in c-Jun activity due to Rapamycin treatment in serum free conditions was attributed to the inhibition of mTOR (Huang et al. 2003). The increased activity of JNK and, c-Jun expression and phosphorylation were shown to be dependent on Rapamycin inhibition of mTOR (Huang et al. 2003). Rapamycin treatment lead to sustained activation of JNK without changing the overall amount of JNK protein (Huang et al. 2003). The treatment of Rapamycin on serum starved Rh30 cells also had no effect on ERK protein levels or activation, suggesting the effect of Rapamycin was specific to JNK pathway (Huang et al. 2003).

These data in Rh30 cells combined with the results seen in C2C12 myoblasts suggest mTOR as a possible area of interest for the regulation of AP-1 protein c-Jun in skeletal muscle myoblasts in the future. In particular, Huang and colleagues (2003) found that in Rapamycin induced activation of JNK pathway which led to increased activity of c-Jun involved 4E-BP1 hypophosphorylation. According to the proposed model pathway, the hypophosphorylation leads to the activation of ASK1 and subsequently the sustained activation of JNK and increased activity of c-Jun (Huang et al. 2003).

Hypophosphorylation of 4E-BP1 is characteristic of quiescent cells as it inhibits eIF4E which is essential for translation (Gingras et al. 1999). As the hypophosphorylation of 4E-BP1 is characteristic of quiescent cells, it is a pathway of interest for mTOR regulation of c-Jun in quiescent satellite cells.

The effect of AP-1 proteins on autophagy is unique to both induction mechanism and the particular protein AP-1 itself. The ectopic expression of JunB had a unique effect on autophagy in comparison to JunB (Figure 9). JunB had no effect on autophagosome content in myoblasts in growth conditions unlike c-Jun. Consequently, in growth conditions the alteration to autophagosome content is specific to ectopic expression of c-Jun and is not a result of general changes to Jun proteins. JunB did appear to have a suppressive effect on LC3II autophagosome content in Rapamycin induced autophagy similar to as seen with ectopic expression of c-Jun. The similar effects of c-Jun and JunB in the suppressive effect on autophagy in myoblasts is like that seen in HEK293 cells during starvation conditions (Yogev et al. 2010).

When the effect of AP-1 proteins on starvation induced autophagy was tested by Yogev and colleagues (2010), only c-Jun and JunB were observed to have a suppressive effect on starvation induced autophagy. It has been shown in C2C12 myotubes ectopic expression of JunB has not had a suppressive effect on autophagy initiation observed through LC3 puncta formation during Forkhead Box O3 (FOXO3) ectopic expression; FOXO3 initiates autophagy through regulation of Beclin-1 and Bcl-2 Interacting Protein 3 (Bnip3)- both which are able to initiate autophagy- or starvation (Raffaello et al. 2010). JunB ectopic expression was also unable to inhibit FOXO3 mediated autophagy progression, marked by the conjugation of LC3 I to LC3 II (Raffaello et al. 2010). JunB did appear to partially inhibit the effect of FOXO3 activation of proteolysis (Raffaello et al. 2010). Raffaello and colleagues (2010) concluded that JunB acted independently of mTOR myotubes. Our results shown in myoblasts suggest that ectopic expression of JunB is similar in growth conditions of myoblasts as myotubes and has no effect on autophagosome content, However, in Rapamycin induced autophagy JunB does appear to have an effect on autophagosome content in myoblasts.

Similar to c-Jun, JunB expression did appear to be altered due to Rapamycin treatment (Figure 9a). However ectopic expression of JunB appeared enhanced in Rapamycin conditions (Figure 9a). The ectopic expression of JunB did have suppressive effects on Rapamycin induced autophagy (Figure 8a). In control conditions JunB expression appeared to be suppressed in Rapamycin treatment compared to growth conditions (Figure 9a). Interestingly JunB has been shown to have no effect on mTOR activity in Hek293 cells in either starvation condition and ectopic JunB expression and has been shown to act independently of the mTOR pathway in C2C12 myotubes (Raffaello et al.

2010; Yogev et al. 2010). In JunB overexpressed HEK293 cells, Rapamycin induced autophagy was inhibited, and mTOR downstream target was unaffected, indicating the repression of Rapamycin induced autophagy in Hek293 cells by JunB is independent of mTOR pathway (Yogev et al. 2010). These findings combined with this study's observation of a repression of JunB in rapamycin induced autophagy in myoblasts suggests that JunB may also interact with mTOR in a similar way in C2C12 myoblasts as in HEK293 cells.

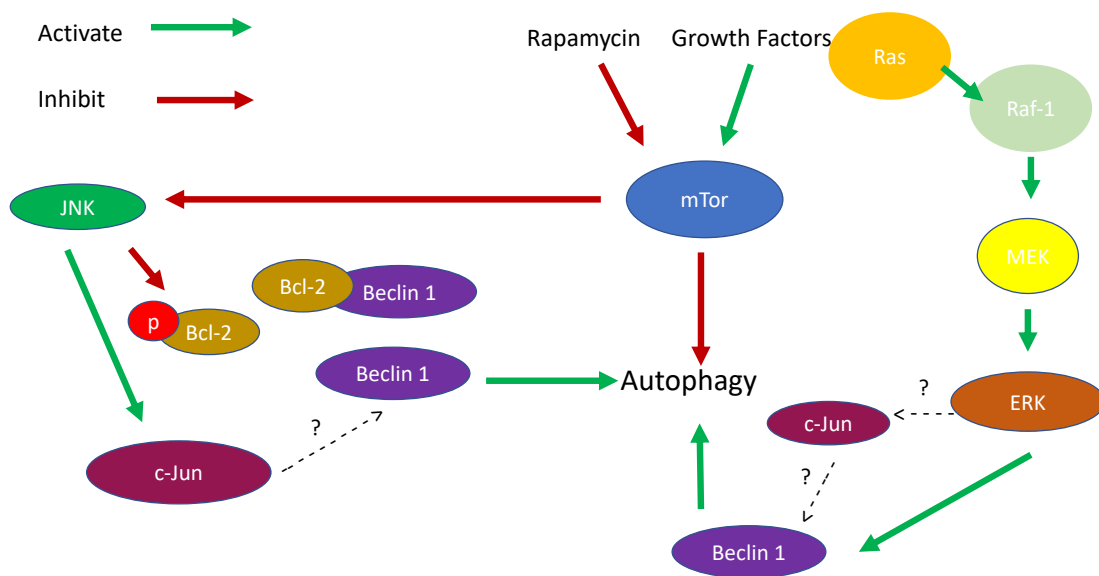


Figure 10: AP-1 protein c-Jun in relation to autophagy induction mechanisms.

A schematic of autophagy induction pathways with inclusion of AP-1 protein c-Jun. Autophagy can be inhibited by mTOR in its activated state. Rapamycin inhibits mTOR leading to the induction of autophagy. During inactivation of mTOR JNK phosphorylation of c-Jun increases. Increased activation of JNK during Rapamycin treatment leads to increase of c-Jun activity which contributes to changes in autophagosome content; c-Jun contributes to changes in autophagy. AP-1 protein c-Jun may have an enhancer effect through up regulation of Beclin-1 or perhaps have a suppressive effect on Beclin-1 expression.

Conclusions and Future Work

AP-1 proteins are a family of transcription factors which are emerging to have roles in various processes of skeletal muscle. This study has shown that AP-1 proteins effect autophagy in both C2C12 myoblasts and primary myogenic progenitor cells. Both the ectopic expression of c-Jun and reduction of endogenous c-Jun in myoblasts in growth conditions leads to an increase LC3II in autophagosomes. In a heterogenous population of Pax7⁺ and Pax7⁻ modification of c-Jun expression lead to a decline in autophagy. Overall it appears that disruption of normal c-Jun expression alters autophagosome content in basal conditions. In C2C12 cells, the relationship between c-Jun and autophagosome content to be non-linear as both reduction and ectopic expression yield the same increases. The non-linear relationship observed in this study suggests that c-Jun could perhaps contribute to the overall autophagy homeostasis of the cell.

Ectopic expression of c-Jun repressed Rapamycin induced increase of LC3II in myoblasts. JunB expression also repressed Rapamycin induced autophagy in myoblast. Ap-1 proteins appear to play a role in the regulation of Rapamycin induced autophagosome content changes. The reduction in Rapamycin induced autophagosome content changes relates back to the previously stated conclusion that c-Jun is perhaps contributing to cellular autophagy homeostasis. High levels of autophagy can lead to autophagy mediated cell death, perhaps in conditions where autophagy is induced to these potentially cellular lethal levels, c-Jun and JunB act to regulate autophagy to maintain homeostatic levels. This regulation of Rapamycin

induced autophagy would be an area for future investigation. JunB has been shown to have no interaction with mTOR in myotubes (Raffaello et al. 2010) and c-Jun has been shown to interact with mTOR in Rh30 cells (Huang et al. 2003), further investigation of this interaction in the context of myogenic progenitors. Rapamycin induction of autophagy has been used previously in studies to aid in the restoration of regenerative capacity of geriatric satellite cells (García-Prat et al. 2016). Understanding of the particular mechanisms Rapamycin targets downstream of mTOR in myogenic progenitor cells may illustrate a more precise way to regulate autophagy in a cell specific manner.

The findings of c-Jun expression in Pax7⁺ satellite cells, along with its apparent effects on both basal and Rapamycin induced changes in autophagosome content make c-Jun a promising candidate for further study. JunB is a protein which warrants further investigation in its potential interaction with the mTOR regulation of autophagy which was previously explored in myotubes (Huang et al. 2003). JunD would also be of interest due to its previously shown presence in myoblast (Andreucci et al. 2002).

Investigating the regulation by c-Jun of protein expression of autophagy critical factors such as LC3, Beclin-1 as well as autophagy inhibiting proteins such as Bcl-2 could shed light onto the mechanism by which c-Jun acts to both promote and inhibit autophagy in myogenic progenitors. As AP-1 proteins are transcription factors it would be critical to find a link between the observed effects and known autophagy relevant genes. Previous studies have primarily used ChIP to show c-Jun binding to promoter regions of autophagy essential genes such as Beclin-1 and LC3 in cancer cells (Li et al. 2009; Sun et al. 2011). It would be beneficial to create a comprehensive list of c-Jun effected

genes in myogenetic progenitor cells — both Pax7⁺ and Pax7⁻ — cells in basal proliferative conditions. As the effect of c-Jun in skeletal muscle appears to be specific to basal or induced autophagy similar to what has as seen in cancer cell lines (Li et al. 2009; Sun et al. 2011). The diverse nature of c-Jun on the regulation of autophagy in other tissue types requires a specific RNA sequencing analysis in either basal conditions or induced conditions to yield a significant amount of data of c-Jun regulated genes. RNA sequencing data of c-Jun knockdown verses c-Jun endogenous expression could then be processed through a GO ontology analysis could be performed on autophagy related genes — both initiation and inhibition known genes of autophagy. These investigations would provide the critical link between observed effects of c-Jun on autophagy and the mechanism by which it regulates both basal and induced autophagy. The same experiment could also be carried out on other AP-1 proteins of interest, with a focus on the factors which are expressed in both Pax7⁺ and Pax7⁻ cells. Evidence suggests that AP-1 proteins, specifically c-Jun have opposing effects dependent of autophagy induction mechanisms. We have shown that c-Jun is an interesting candidate for further study in the regulation of autophagy in myogenic progenitor cells. Understanding the mechanisms which regulate autophagy in skeletal muscle progenitor cells aids in understanding the maintenance of quiescence in satellite cells and, the preservation of injury response capabilities in aged individuals and disease models.

References

Alli NS, Yang EC, Miyake T, Aziz A, Collins-Hooper H, Patel K, McDermott JC. 2013 Signal-dependent fra-2 regulation in skeletal muscle reserve and satellite cells. *Cell death & disease*. 4(6):e692.

Andreucci JJ, Grant D, Cox DM, Tomc LK, Prywes R, Goldhamer DJ, Rodrigues N, Bédard PA, McDermott JC. 2002 Composition and function of AP-1 transcription complexes during muscle cell differentiation. *Journal of Biological Chemistry*. 277(19):16426-32. doi:10.1074/jbc.M110891200.

Angel P, Hattori K, Smeal T, Karin M. 1988. The jun proto-oncogene is positively autoregulated by its product, Jun/AP-1. *Cell*. 55(5):875-85. doi:10.1016/0092-8674(88)90143-2

Angel P, Karin M. 1991. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*. 1072(2-3):129-57. doi:10.1016/0304-419X(91)90011-9.

Bakiri L, Lallemand D, Bossy-Wetzel E, Yaniv M. 2000. Cell cycle-dependent variations in c-Jun and JunB phosphorylation: a role in the control of cyclin D1 expression. *The EMBO journal*. 2000. 19(9):2056-68. doi:10.1093/emboj/19.9.2056.

Bechet D, Tassa A, Taillandier D, Combaret L, Attaix D. 2005 Lysosomal proteolysis in skeletal muscle. *The international journal of biochemistry & cell biology*. 37(10):2098-114. doi:10.1016/j.biocel.2005.02.029

Bengal E, Ransone LJ, Scharfmann R, Dwarki VJ, Tapscott SJ, Weintraub H, Verma IM. 1992. Functional antagonism between c-Jun and MyoD proteins: a direct physical association. *Cell*. 68(3):507-19. doi:10.1016/0092-8674(92)90187-H.

Bentzinger CF, Wang YX, Rudnicki MA. 2012. Building muscle: molecular regulation of myogenesis. *Cold Spring Harbor perspectives in biology*. 4(2):a008342. doi:10.1101/cshperspect.a008342.

Bober E, Franz T, Arnold HH, Gruss P, Tremblay P. 1994. Pax-3 is required for the development of limb muscles: a possible role for the migration of dermomyotomal muscle progenitor cells. *Development*. 120(3):603-12.

Brown JR, Nigh E, Lee RJ, Ye H, Thompson MA, Saudou F, Pestell RG, Greenberg ME. Fos family members induce cell cycle entry by activating cyclin D1. *Molecular and cellular biology*. 1998 Sep 1;18(9):5609-19.doi: 10.1128/MCB.18.9.5609

Buckingham M, Bajard L, Chang T, Daubas P, Hadchouel J, Meilhac S, Montarras D, Rocancourt D, Relaix F. 2003. The formation of skeletal muscle: from somite to limb. *Journal of anatomy*. 202(1):59-68. doi:10.1046/j.1469-7580.2003.00139.x.

Chal J, Pourquié O. 2017. Making muscle: skeletal myogenesis in vivo and in vitro. *Development*. 144(12):2104-22.doi: 10.1242/dev.151035

Cheung TH, Rando TA. 2013. Molecular regulation of stem cell quiescence. *Nature reviews Molecular cell biology*. (6):329. doi:10.1038/nrm3591.

Chi N, Epstein JA. 2002. Getting your Pax straight: Pax proteins in development and disease. *TRENDS in Genetics*.18(1):41-7.doi: 10.1016/S0168-9525(01)02594-X

Chinenov Y, Kerppola TK. 2001. Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity. *Oncogene*. 20(19):2438. doi: 10.1038/sj.onc.1204385

Chiu R, Angel P, Karin M. 1989. Jun-B differs in its biological properties from, and is a negative regulator of, c-Jun. *Cell*. 59(6):979-86. doi:10.1016/0092-8674(89)90754-X.

Collins CA, Olsen I, Zammit PS, Heslop L, Petrie A, Partridge TA, Morgan JE. 2005. Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. *Cell*. 122(2):289-301.doi: 10.1016/j.cell.2005.05.010

Dietrich S, Abou-Rebyeh F, Brohmann H, Bladt F, Sonnenberg-Riethmacher E, Yamaai T, Lumsden A, Brand-Saberi B, Birchmeier C. 1999. The role of SF/HGF and c-Met in the development of skeletal muscle. *Development*. 126(8):1621-9.

Edom-Vovard F, Bonnin MA, Duprez D. 2001. Misexpression of Fgf-4 in the chick limb inhibits myogenesis by down-regulating Fek expression. *Developmental biology*. 233(1):56-71. doi:10.1006/dbio.2001.0221.

Erbay E, Chen J. 2001. The mammalian target of rapamycin regulates C2C12 myogenesis via a kinase-independent mechanism. *Journal of Biological Chemistry*. 276(39):36079-82. doi:10.1074/jbc.C100406200

Eskelinen EL, Tanaka Y, Saftig P. 2003. At the acidic edge: emerging functions for lysosomal membrane proteins. *Trends in cell biology*. 13(3):137-45. doi:10.1016/S0962-8924(03)00005-9.

Fass E, Shvets E, Degani I, Hirschberg K, Elazar Z. 2006. Microtubules support production of starvation-induced autophagosomes but not their targeting and fusion with lysosomes. *Journal of Biological Chemistry*. 281(47):36303-16. doi:10.1074/jbc.M607031200.

Fiacco E, Castagnetti F, Bianconi V, Madaro L, De Bardi M, Nazio F, D'Amico A, Bertini E, Cecconi F, Puri PL, Latella L. 2016. Autophagy regulates satellite cell ability to regenerate normal and dystrophic muscles. *Cell death and differentiation*. 23(11):1839–49. doi:10.1038/cdd.2016.70.

Fortini P, Ferretti C, Iorio E, Cagnin M, Garribba L, Pietraforte D, Falchi M, Pascucci B, Baccarini S, Morani F, Phadngam S. 2016. The fine tuning of metabolism, autophagy and differentiation during in vitro myogenesis. *Cell death & disease*. 7(3):e2168. doi:10.1038/cddis.2016.50.

Furuta S, Hidaka E, Ogata A, Yokota S, Kamata T. 2004 Ras is involved in the negative control of autophagy through the class I PI3-kinase. *Oncogene*. 23(22):3898. doi:10.1038/sj.onc.1207539.

García-Prat L, Martínez-Vicente M, Perdiguero E, Ortet L, Rodríguez-Ubreva J, Rebollo E, Ruiz-Bonilla V, Gutarra S, Ballestar E, Serrano AL, Sandri M. 2016. Autophagy maintains stemness by preventing senescence. *Nature*. 529(7584):37. doi:10.1038/nature16187.

Gibson, Marcia C., and Edward Schultz. 1983. Age-related Differences in Absolute Numbers of Skeletal Muscle Satellite Cells. *Muscle & Nerve*. 6(8):574-80. doi:10.1002/mus.880060807.

Gingras AC, Gygi SP, Raught B, Polakiewicz RD, Abraham RT, Hoekstra MF, Aebersold R, Sonenberg N. 1999. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. *Genes & development*. 13(11):1422-37.

Gruda MC, Kovary K, Metz R, Bravo R. 1994. Regulation of Fra-1 and Fra-2 phosphorylation differs during the cell cycle of fibroblasts and phosphorylation in vitro by MAP kinase affects DNA binding activity. *Oncogene*. 1994 Sep;9(9):2537-47. doi: 10.1016/j.socscimed.2012.04.042.

Halazonetis TD, Georgopoulos K, Greenberg ME, Leder P. 1988. c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities. *Cell*. 55(5):917-24. doi:10.1016/0092-8674(88)90147-X.

Ham J, Babij C, Whitfield J, Pfarr CM, Lallemand D, Yaniv M, Rubin LL. 1995. A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death. *Neuron*. 14(5):927-39. doi:10.1016/0896-6273(95)90331-3.

Han J, Jiang Y, Li Z, Kravchenko VV, Ulevitch RJ. 1997. Activation of the transcription factor MEF2C by the MAP kinase p38 in inflammation. *Nature*. 386(6622):296. doi:10.1038/386296a0.

Han TH, Prywes R. 1995. Regulatory role of MEF2D in serum induction of the c-jun promoter. *Molecular and cellular biology*. 15(6):2907-15. doi:10.1128/MCB.15.6.2907.

He C, Klionsky DJ. 2009. Regulation mechanisms and signaling pathways of autophagy. *Annual review of genetics*. 43:67-93. doi:10.1146/annurev-genet-102808-114910.

Hindi L, McMillan JD, Afroze D, Hindi SM, Kumar A. 2017. Isolation, culturing, and differentiation of primary myoblasts from skeletal muscle of adult mice. *Bio-protocol*. 7(9). 10.21769/BioProtoc.2248

Hirsinger E, Duprez D, Jouve C, Malapert P, Cooke J, Pourquié O. 1994. Noggin acts downstream of Wnt and Sonic Hedgehog to antagonize BMP4 in avian somite patterning. *Development*. 124(22):4605-14.

Horst D, Ustanina S, Sergi C, Mikuz G, Juergens H, Braun T, Vorobyov E. 2003. Comparative expression analysis of Pax3 and Pax7 during mouse myogenesis. *International journal of developmental biology*. 50(1):47-54. doi:10.1387/ijdb.052111dh.

Hosokawa N, Hara T, Kaizuka T, Kishi C, Takamura A, Miura Y, Iemura SI, Natsume T, Takehana K, Yamada N, Guan JL. 2009. Nutrient-dependent mTORC1 association with

the ULK1–Atg13–FIP200 complex required for autophagy. *Molecular biology of the cell*. 20(7):1981-91. doi:10.1091/mbc.E08-12-1248.

Huang S, Shu L, Dilling MB, Easton J, Harwood FC, Ichijo H, Houghton PJ. 2003. Sustained activation of the JNK cascade and rapamycin-induced apoptosis are suppressed by p53/p21Cip1. *Molecular cell*. 11(6):1491-501.

Hutcheson DA, Zhao J, Merrell A, Haldar M, Kardon G. 2009. Embryonic and fetal limb myogenic cells are derived from developmentally distinct progenitors and have different requirements for β -catenin. *Genes & development*. 23(8):997-1013. doi:10.1101/gad.1769009

Itakura E, Kishi C, Inoue K, Mizushima N. 2008. Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. *Molecular biology of the cell*. 19(12):5360-72. doi:10.1091/mbc.E08-01-0080.

Johnson RS, Van Lingen B, Papaioannou VE, Spiegelman BM. 1993. A null mutation at the c-jun locus causes embryonic lethality and retarded cell growth in culture. *Genes & development*. 7(7b):1309-17. doi:10.1101/gad.7.7b.1309.

Karin M. 1996. The Regulation of AP-1 Activity by Mitogen-Activated Protein Kinases. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 351(1336):127-34. doi:10.1098/rstb.1996.0008.

Kato Y, Kravchenko VV, Tapping RI, Han J, Ulevitch RJ, Lee JD. 1997. BMK1/ERK5 regulates serum-induced early gene expression through transcription factor MEF2C. *The EMBO journal*. 16(23):7054-66. doi:10.1093/emboj/16.23.7054

Klionsky, DJ. 2007. Autophagy: From Phenomenology to Molecular Understanding in Less than a Decade. *Nature Reviews Molecular Cell Biology*. (11):931-7. doi:10.1038/nrm2245.

Kochetkova EY, Blinova GI, Zubova SG, Bykova TV, Pospelov VA, Pospelova TV. 2017. The MEK/ERK pathway is essential for maintenance of cytoprotective autophagy in E1A+ cHA-RAS transformants after exposure to radiation. *Cell and Tissue Biology*. 11(4):268-74.

Kouzarides T, and Ziff E. 1988. The Role of the Leucine Zipper in the Fos-Jun Interaction. *Nature*. doi:10.1038/336646a0.

Kuma A, Hatano M, Matsui M, Yamamoto A, Nakaya H, Yoshimori T, Ohsumi Y, Tokuhisa T, Mizushima N. 2004. The role of autophagy during the early neonatal starvation period. *Nature*. 432(7020):1032. doi:10.1038/nature03029.

Lee YK, Lee JA. 2016. Role of the mammalian ATG8/LC3 family in autophagy: differential and compensatory roles in the spatiotemporal regulation of autophagy. *BMB reports*. 49(8):424.

Lepper C, Partridge TA, Fan CM. 2011. An absolute requirement for Pax7-positive satellite cells in acute injury-induced skeletal muscle regeneration. *Development*. 138(17):3639-46. doi:10.1242/dev.067595.

Li DD, Wang LL, Deng R, Tang J, Shen Y, Guo JF, Wang Y, Xia LP, Feng GK, Liu QQ, Huang WL. 2009. The pivotal role of c-Jun NH2-terminal kinase-mediated Beclin 1 expression during anticancer agents-induced autophagy in cancer cells. *Oncogene*. 28(6):886. doi:10.1038/onc.2008.441.

Matsunaga K, Saitoh T, Tabata K, Omori H, Satoh T, Kurotori N, Maejima I, Shirahama-Noda K, Ichimura T, Isobe T, Akira S. 2009. Two Beclin 1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages. *Nature cell biology*. 11(4):385. doi:10.1038/ncb1846.

McMillan EM., and Quadrilatero J. 2014. Autophagy Is Required and Protects against Apoptosis during Myoblast Differentiation. *Biochemical Journal*. doi:10.1042/BJ20140312.

Mehrpour M, Esclatine A, Beau I, Codogno P. 2010. Overview of macroautophagy regulation in mammalian cells. *Cell research*. 20(7):748. doi:10.1038/cr.2010.82.

Murphy M, Kardon G. 2011. Origin of vertebrate limb muscle: the role of progenitor and myoblast populations. *Current topics in developmental biology*. (96):1-32. doi:10.1016/B978-0-12-385940-2.00001-2.

Nakatogawa H, Suzuki K, Kamada Y, Ohsumi Y. 2009. Dynamics and diversity in

autophagy mechanisms: lessons from yeast. *Nature reviews Molecular cell biology*. 10(7):458. doi:10.1038/nrm2708.

Ogata M, Hino SI, Saito A, Morikawa K, Kondo S, Kanemoto S, Murakami T, Taniguchi M, Tanii I, Yoshinaga K, Shiosaka S. 2006. Autophagy is activated for cell survival after endoplasmic reticulum stress. *Molecular and cellular biology*. 26(24):9220-31. doi:10.1128/MCB.01453-06.

Olguin HC, Yang Z, Tapscott SJ, Olwin BB. 2007. Reciprocal inhibition between Pax7 and muscle regulatory factors modulates myogenic cell fate determination. *J Cell Biol*. 177(5):769-79. doi:10.1083/jcb.200608122.

Olivera-Martinez I, Coltey M, Dhouailly D, Pourquié O. 2000. Mediolateral somitic origin of ribs and dermis determined by quail-chick chimeras. *Development*. 127(21):4611-7.

Ordahl CP, Le Douarin NM. 1992. Two myogenic lineages within the developing somite. *Development*. 114(2):339-53. doi:10.1007/978-94-017-7432-1_7.

Otto A, Schmidt C, Patel K. 2006. Pax3 and Pax7 expression and regulation in the avian embryo. *Anatomy and embryology*. 211(4):293-310. doi:10.1007/s00429-006-0083-3.

Pattingre S, Bauvy C, Codogno P. 2003. Amino acids interfere with the ERK1/2-dependent control of macroautophagy by controlling the activation of Raf-1 in human colon cancer HT-29 cells. *Journal of Biological Chemistry*. doi:10.1074/jbc.M210998200.

Pattingre S, Bauvy C, Carpentier S, Levade T, Levine B, Codogno P. 2009. Role of JNK1-dependent Bcl-2 phosphorylation in ceramide-induced macroautophagy. *Journal of Biological Chemistry*. 284(5):2719-28. doi:10.1074/jbc.M805920200.

Raffaello A, Milan G, Masiero E, Carnio S, Lee D, Lanfranchi G, Goldberg AL, Sandri M. 2010. JunB transcription factor maintains skeletal muscle mass and promotes hypertrophy. *The Journal of cell biology*. 191(1):101-13. doi:10.1083/jcb.201001136.

Relaix F, Montarras D, Zaffran S, Gayraud-Morel B, Rocancourt D, Tajbakhsh S, Mansouri A, Cumano A, Buckingham M. 2006. Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. *J Cell Biol.* 172(1):91-102. doi:10.1083/jcb.200508044.

Rubinsztein DC. 2006. The roles of intracellular protein-degradation pathways in neurodegeneration. *Nature.* 443(7113):780. doi:10.1038/nature05291.

Rubinsztein DC, Gestwicki JE, Murphy LO, Klionsky DJ. 2007. Potential therapeutic applications of autophagy. *Nature reviews Drug discovery.* (4):304. doi:10.1038/nrd2272.

Ryseck RP, Bravo R. 1991. c-JUN, JUN B, and JUN D differ in their binding affinities to AP-1 and CRE consensus sequences: effect of FOS proteins. *Oncogene.* 6(4):533-42.

Schütte J, Viallet J, Nau M, Segal S, Fedorko J, Minna J. 1989. jun-B inhibits and c-fos stimulates the transforming and trans-activating activities of c-jun. *Cell.* 59(6):987-97. doi:10.1016/0092-8674(89)90755-1.

Seale P, Sabourin LA, Girgis-Gabardo A, Mansouri A, Gruss P, Rudnicki MA. 2000. Pax7 is required for the specification of myogenic satellite cells. *Cell.* 102(6):777-86. doi:10.1016/S0092-8674(00)00066-0.

Seglen PO, Berg TO, Blankson H, Fengsrud M, Holen I, Strømhaug PE. Structural aspects of autophagy. In *Intracellular protein catabolism 1996* (pp. 103-111). Springer, Boston, MA.

Shaulian E, Schreiber M, Piu F, Beeche M, Wagner EF, Karin M. 2000. The mammalian UV response: c-Jun induction is required for exit from p53-imposed growth arrest. *Cell.* 103(6):897-908. doi:10.1016/S0092-8674(00)00193-8.

Shaulian E, Karin M. 2002. AP-1 as a regulator of cell life and death. *Nature cell biology.* 4(5):E131. doi:10.1038/ncb0502-e131.

Stokoe D, Stephens LR, Copeland T, Gaffney PR, Reese CB, Painter GF, Holmes AB, McCormick F, Hawkins PT. 1997. Dual role of phosphatidylinositol-3, 4, 5-trisphosphate

in the activation of protein kinase B. *Science*. 277(5325):567-70.
doi:10.1126/science.277.5325.567.

Sui X, Chen R, Wang Z, Huang Z, Kong N, Zhang M, Han W, Lou F, Yang J, Zhang Q, Wang X. 2013. Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment. *Cell death & disease*. 4(10):e838.

Sun T, Li D, Wang L, Xia L, Ma J, Guan Z, Feng G, Zhu X. c-Jun NH2-terminal kinase activation is essential for up-regulation of LC3 during ceramide-induced autophagy in human nasopharyngeal carcinoma cells. *Journal of translational medicine*. 2011 Dec;9(1):161.doi:10.1186/1479-5876-9-161

Szabowski A, Maas-Szabowski N, Andrecht S, Kolbus A, Schorpp-Kistner M, Fusenig NE, Angel P. 2000. c-Jun and JunB antagonistically control cytokine-regulated mesenchymal–epidermal interaction in skin. *Cell*. 103(5):745-55. doi:10.1016/S0092-8674(00)00178-1.

Tanaka Y, Guhde G, Suter A, Eskelinen EL, Hartmann D, Lüllmann-Rauch R, Janssen PM, Blanz J, Von Figura K, Saftig P. 2000. Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice. *Nature*. 406(6798):902.
doi:10.1038/35022595.

Trouche D, Grigoriev M, Lenormand JL, Robin P, Leibovitch SA, Sassone-Corsi P, Harel-Bellan A. 1993. Repression of c-fos promoter by MyoD on muscle cell differentiation. *Nature*. 363(6424):79. doi:10.1038/363079a0.

Wang J, Whiteman MW, Lian H, Wang G, Singh A, Huang D, Denmark T. 2009. A non-canonical MEK/ERK signaling pathway regulates autophagy via regulating Beclin 1. *Journal of Biological Chemistry*. jbc-M109.doi: 10.1074/jbc.M109.026013

Yogev O, Goldberg R, Anzi S, Yogev O, Shaulian E. 2010. Jun proteins are starvation-regulated inhibitors of autophagy. *Cancer research*.:0008-5472.doi: 10.1158/0008-5472.CAN-09-3408

Yin H, Price F, Rudnicki MA. 2013. Satellite cells and the muscle stem cell niche. *Physiological reviews*. 93(1):23-67. doi:10.1152/physrev.00043.2011.

Zammit PS, Relaix F, Nagata Y, Ruiz AP, Collins CA, Partridge TA, Beauchamp JR. 2006. Pax7 and myogenic progression in skeletal muscle satellite cells. *Journal of cell science*. 119(9):1824-32. doi:10.1242/jcs.02908.

Appendix: Extended Materials and Methods

Primary Skeletal Muscle Isolation and Pre-Plating

The following protocol is adapted from Hindi et. al 2017.

70% Ethanol

700mL 100% ethanol

300mL ddH₂O

10% Matrigel

1. Thaw Matrigel in fridge overnight
2. Dissolve 10mL cold Matrigel into 90mL cold DMEM
3. Aliquot 10% Matrigel into tubes and store at -20°C

10% Matrigel Plate

1. Thaw frozen 10% Matrigel on ice or in fridge overnight
2. Place plates and pipette tips on ice
3. Place thawed 10% Matrigel on ice
4. In Biosafety Cabinet coat plates with Matrigel on ice, remove excess
5. Place lids on plates and into incubator to dry- Matrigel will solidify when dry

Collagenase II

1.5 U/mL collagenase D (Boehringer Mannheim Corp.) from -20°C frozen aliquot

2.4 U/mL dispase II (Boehringer Mannheim Corp.) from -20°C frozen aliquot

2.5 mM CaCl₂ added immediately before use

Combine and use immediately or aliquot and store at -20°C without

F-10-based primary myoblast growth medium (PMGM)

400 mL Hams F-10 nutrient mixture (GIBCO)

100 mL fetal calf serum (HyClone) (20% final)

10ng/mL of basic fibroblast growth factor (human; Promega Corp.)

5 mL penicillin/streptomycin (1% final) (Invitrogen)

Store at 4°C

Trypsin

Dilute Trypsin to 2.5% (v/v) in sterile PBS

Overall Primary Skeletal Muscle Isolation Protocol

1. Sacrifice 1-5 CD-1 neonatal mice by approved Animal Care protocol
2. Rinse limbs with 70% ethanol, remove hindlimb muscles
3. Place tissue in 10cm plate containing PBS to keep tissue moist
4. Wash tissue 2X with warm PBS
5. Aspirate PBS
6. Add 2mL (adjust volume for additional mice) warmed collagenase II to tissue and mince with sterile blades

7. Pipette collagenase and tissue mixture into sterile Eppendorf
8. Incubate mixture at 37 °C for 1 hour (or until mixture appears like a slurry)
shaking every 10 minutes and vortexing 30 minutes in for 10 seconds
9. Spin Eppendorf continuing digested tissue for 5 minutes at 350 x g
10. Carefully aspirate supernatant
11. Resuspend pellet in 10mL PMGM
12. Pull mixture up and down in plastic pipette 20-30 times
13. Place resuspended cells onto 10% Matrigel coated plate- let incubate for at 37 °C
48-72 hours until cells have settled
14. Aspirate media
15. Add trypsin to plate, place in 37 °C for 3 minutes
16. Check cells to see if lifted from plate, tap plate on hard surface to remove
remaining cells- all cells should be dissociated from plate
17. Add 10mL warmed PMGM to plate, pooling cells
18. Collect pooled cells and place on uncoated plate
19. Let incubate for 40-60 minutes- allowing fibroblasts to adhere from plate
20. Collect media and suspended cells from plate and transfer onto 10% Matrigel
coated plate
21. Incubate plate for 24-48 hours and repeat steps 14-20 an additional 2-3 times
until plate appears to contain mainly small round myoblast cells and only a small
population of large fibroblast cells
22. Maintain cells in PMGM and refresh every 48 hours

Polyethyleneimine Transfection in C2C12 cells

PEI

Dilute PEI in ddH₂O for concentration of 1µg/µL, store at -20 for long term storage.

1. Day prior to transfection cells were observed to be at 50% confluency in 6-well or 10cm dish
2. Refresh cells with fresh growth media 3 hours prior to transfection
3. Add 200µL room temperature DMEM to 1.5mL Eppendorf tubes
4. Dilute 3µg/7µg (6 well/10cm plate) of DNA plasmid with 9µg/21µg PEI (6 well/10cm) in the DMEM Eppendorf tubes
5. Incubate at room temperature for 20 minutes
6. Add DNA/PEI complex to cells
7. Harvest cells 48 hours post-transfection

Calcium Phosphate (CaPO₄) siRNA Transfection in C2C12 cells

2x HEBES Solution pH 7.15

16.4g NaCl

11.9g HEPES

0.21g NA₂HPO₄

700mL ddH₂O

2.5M CaCl₂

36.76g CaCl₂—2 H₂O

100ml ddH₂O

CaPO₄ Overall Protocol

8. Day prior to transfection cells were observed to be at 40-50% confluency in 6-well dish
9. Refresh cells with fresh growth media 3 hours prior to transfection
10. For each plate being transfected mix together: 100nM siRNA or Scramble universal control, 1.25µg dsRed plasmid, 3.75µg pBSK, 10µL CaCl₂ and sterile ddH₂O up to 100µL.
11. Add siRNA-DNA mixture dropwise to 100µL 2XHEBES while vortexing
12. Incubate mixture for 20-30 minutes at room temperature
13. Remove 200µL from plates
14. Add 200µL of transfection mixture to each plate- let transfect for approximately 16-20 hours
15. Refresh growth media
16. Harvest 48 hours post transfection

Lipofectamine 2000 Transfection Protocol of Primary Skeletal Muscle

F-10-based primary myoblast growth medium (PMGM)

400 mL Hams F-10 nutrient mixture (GIBCO)

100 mL fetal calf serum (HyClone) (20% final)

10ng/mL of basic fibroblast growth factor (human; Promega Corp.)

5 mL penicillin/streptomycin (1% final) (Invitrogen)

Store at 4°C

Opti-MEM (GIBCO)

Overall Protocol

1. Day prior to transfection cells were observed to be at 40-50% confluency in 6-well dish
2. Refresh cells with fresh growth media 3 hours prior to transfection. For each plate being transfected mix together: 4 μ g pcDNA or HA-cJun with 200mL Opti-mem. In another Eppendorf mix 200mL Opti-mem with 6 μ L Lipofectamine.
3. Combine diluted DNA with diluted Lipofectamine and incubate for 5 minutes at room temperature.
4. While DNA:Lipofectamine complex incubates aspirate media from cells and refresh with 500mL Opti-mem media warmed to 37 °C
5. Add DNA:Lipofectamine complex to cells, incubate for 4-6 hours
6. Add 1.5mL fresh PMGM to cells

7. Harvest or fix cells 48 hours post transfection

Western Blot Analysis Protocol

Wash Buffer

1X TBST- TBS with 0.5% tween20 for antibodies diluted in TBST (Cell signalling antibodies and actin)

1XPBST- PBS with 0.5% tween20 for antibodies diluted in PBST (Santa Cruz antibodies)

10X Transfer Buffer

288g glycine

64.4g Tris base

1.8L ddH₂O

Transfer Buffer

100mL of 10X transfer buffer

100mL methanol

800mL of ddH₂O

Blocking Buffer

150 mL 1X TBST Wash Buffer

7.5g non-fat powder milk

Antibody Dilutions- 5mL

Antibodies were diluted into 1:1000 dilution with final volume of 5mL. Antibodies were diluted into either 5% milk in TBST (0.5% tween) or 5% milk in PBST(0.5% tween).

Cell Protein Extracts

NP40 Lysis Buffer

1.25mL of 1M Tris (pH8)

0.75mL of 5M NaCl

1.25mL of 10%NP-40

100 μ L of 0.5M EDTA

5mL of NaF

2.5mL of Sodium Pyrophosphate

Add ddH₂O to reach 25mL

To 2mL of NP-40 Lysis Buffer add:

20 μ L of 0.1M Na₃VO₄

20 μ L of 100 mM PMSF

0.2 μ L of 10 μ g/ μ L Leupeptin

0.2 μ L of 10 μ g/ μ L Apotinin

0.5 μ L of 5 μ g/ μ L Pepstatin A

1. Remove cells from incubator and place on ice
2. Aspirate media and wash cells 3x with 5mL cold 1XPBS
3. Aspirate 1xPBS
4. Add 1mL 1XPBS to plate
5. Scrape cells off plate and rinse scraper in ddH₂O between plates
6. Pipette 1mL PBS with plate contents into 1.5mL Eppendorf, place on ice
7. Spin down at max speed for 15-20 seconds
8. Discard supernatant
9. Resuspend pellet in approximately 70µL lysis buffer and protease inhibitor combination
10. Vortex every 10 minutes for 30 seconds (on ice) for a total of 40 minutes
11. Store in -80°C or use immediately

Sample preparation

Following whole cell lysis and protein extraction protein concentration was determined using a Bradford Assay.

1. Samples contain 20ug protein with 3X Lamelli buffer at a (v/v protein) of 50%
Lamelli buffer
2. Boil samples for 5 minutes at 95°C
3. Spin down samples at max speed for 5-10 seconds
4. Run samples in gel or store at -20°C until ready

Western Blot Overall Protocol

1. Load gels with 5 μ L ladder and samples
2. Run gel at 150V in 1X Lamelli buffer for approximately an hour
3. Following completion of gel run activate membrane in 95% methanol for 5 minutes
4. Wet all Waterford paper filters and sponges in 1X transfer buffer
5. Wash membrane and gel in transfer buffer
6. Assemble transfer sandwich; black anode plastic, sponge, filter paper, gel, membrane, filter paper, sponge, red cathode plastic
7. Place sandwich in transfer buffer, and place icepack in running tank; run at 100V for an hour or 30V overnight in 4°C room
8. Remove membrane from sandwich and rinse in wash buffer
9. Block membrane in blocking buffer for 1 hour
10. Place membrane in container with primary antibody, rock for 1 hour at room temperature or overnight at 4°C
11. Wash membrane 3X for 5 minutes each wash
12. Place membrane in new container with secondary antibody, rock for 1 hour at room temperature
13. Wash membrane 3X for 5 minutes each wash
14. Add 500 μ L of ECL chemiluminescent to membrane, incubate for 1 minute
15. Remove excess chemiluminescent and put membrane in between plastic sheet

16. Place film on top of membrane in dark room
17. Waiting time for exposure depends on strength of signal
18. Fix and develop film

Skeletal Muscle Fiber Isolation

0.2% Collagenase type I

Dilute Collagenase type I to 0.2% concentration in DMEM. Make 1mL aliquots- 1 aliquot can be used per mouse. Store at -20 for extended storage. Warm to 37°C in water-bath prior to use.

Isolation Media

20% FBS in DMEM warmed to 37°C

Culture Media

DMEM with 20% FBS and 1% Chicken Embryo extract.

Coated Dishes/ Pipettes

Coat several dishes with 10% Horse Serum (HS) in DMEM

1. Add 10% HS to dish, swirl around dish and coat sides
2. Remove excess

Coat large bore pipettes with 10% HS

1. Pipette 10% HS up and down in glass pipette several times

2. Allow pipette excess HS to run out of pipette by standing in large Eppendorf

Overall protocol (For One Mouse)

1. Sacrifice 6-8 week old male mouse in accordance to Animal Care protocol
2. Spray hindlimbs and tools with 70% Ethanol
3. Dissect back hair and skin from hind limbs. Spray with additional ethanol to dampen hair to assist with removal
4. Remove EDL in one piece by cutting first the distal tendon followed by proximal tendon- allows for the least amount of injury
5. Place muscle into warmed Collagenase solution
6. Repeat steps 4-5 with other hind limb
7. Incubate muscle in Collagenase solution for approximately 1 hour, shaking lightly every 15 minutes- muscle bundle should seem loosened at end of incubation period
8. Transfer loosened muscle bundle to coated dish containing enough isolation media that fibers are moist
9. Flush muscle with warm media until fibers begin to release- this can be done under dissection microscope to aid with visualization of dissociated fibers
10. Incubate for 30 minutes-1hr in 37°C cell culture incubator
11. Remove twisted, coiled dead fibers
12. Wash remaining fibers 2X with warmed PBS then aspirate using a 30G needle and syringe aids with aspiration

13. Add enough culture media to keep fibers in suspension, incubate for approximately 3-4 hours to allow fibers to recover and satellite cells to begin to activate
14. Wash fibers 2X with PBS before fixation and further staining